INHERITED AND ACQUIRED GENETIC FACTORS IN MYELOPROLIFERATIVE NEOPLASMS

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Submitted by

Dr. Damla Olcaydu Kraucher

Supervisor:
Robert Kralovics, Ph.D.

Center of Molecular Medicine, Austrian Academy of Sciences
Lazarettgasse 14, AKH BT25.3, 1090 Vienna

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Abbreviations

A	Adenosine
ABL	V-abl Abelson murine leukemia viral oncogene homolog 1
AML	Acute myeloid leukemia
Bcl-x_L	B-cell leukemia/lymphoma XL
BCR	Breakpoint cluster region
BFU-E	Burst-forming unit erythroid
C	Cytosine
CI	Confidence interval
CML	Chronic myeloid leukemia
D, del	Deletion
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotidetriphosphate
EEC	Endogenous erythroid colony
EPO	Erythropoietin
EPOR	Erythropoietin receptor
ET	Essential thrombocythemia
F	Phenylalanine
FAM	6-carboxyfluorescein
FISH	Fluorescent in situ hybridization
Flt3-L	Flt3 (fms-like tyrosine kinase receptor-3) ligand
G	Guanin
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-monocyte colony stimulating factor
GCSFR	Granulocyte colony stimulating factor receptor
HPLC	High-performance liquid chromatography
IGF-1	Insulin-like growth factor 1
IL-3	Interleukin 3
IL-6	Interleukin 6
IRR	Incidence rate ratio
JAK2	Janus Kinase 2
JH2	JAK homology 2 domain
K	Lysine
kb	kilo base pairs
L	Leucine
LOH	Loss of heterozygosity
Mb	mega base pairs
MAPK	Mitogen activated protein kinase
MDS	Myelodysplastic syndrome
MPL	Myeloproliferative leukemia virus oncogene
MPN	Myeloproliferative neoplasm
mRNA	Messenger RNA
NFIB	Nuclear factor I-B
OR	Odds ratio
p	Short arm of chromosome
PBMNC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
Ph	Philadelphia chromosome

PI3K	Phosphoinositol 3-kinase
PLT	Platelet count
PMF	Primary Myelofibrosis
PMN	Polymorphnuclear cells
PRV-1	Polycythemia rubra vera 1 protein
PTP-Meg2	Protein tyrosine phosphatase Meg2
PV	Polycythemia vera
PVSG	Polycythemia Vera Study Group
q	Long arm of chromosome
RNA	Ribonucleic acid
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
T	Thymine
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TPO	Thrombopoietin
V	Valine
W	Tryptophan
WHO	World Health Organization
wt	Wild type

Abstract in English

Myeloproliferative neoplasms (MPN) are a heterogeneous group of myeloid malignancies that share the main pathogenetic feature of clonal hematopoiesis. Acquired genetic defects such as point mutations or chromosomal deletions have been implicated in aberrant proliferation and clonal outgrowth of hematopoietic cells. In this study, we aimed to elucidate the role of recurrent genetic aberrations in disease pathogenesis of MPN. Although deletions of the long arm of chromosome 13 (del13q) have previously been reported in myeloid neoplasms, their actual frequency and pathogenetic impact in MPN remains unknown. Using high-resolution microarray mapping analysis, we identified a total number of 16 patients with del13q and determined a frequency of about 3% in MPN. The delineation of the deleted regions revealed a novel common deleted region of about 850 kb that contains 7 genes, including the Retinoblastoma 1 (*RB1*) tumor suppressor gene. Further genetic and functional analysis did not show a role for the monoallelic loss of *RB1* in MPN pathogenesis. However, data obtained from the analysis of clonal hierarchies of del13q and other genetic defects in hematopoietic progenitor cells did suggest that the JAK2-V617F mutation could be acquired multiple times in the same individual. Further evidence for multiple acquisitions of JAK2-V617F could be gained by a PCR-based assay, revealing that about 3% of MPN patients acquired the JAK2-V617F mutation at least two times. This analysis further showed that a certain allele of the *JAK2* gene was more susceptible to acquire the V617F as well as exon 12 mutations. Subsequently, we identified a common haplotype – the *JAK2* GGCC haplotype – that harbors the *JAK2* gene and confers susceptibility to *JAK2* mutation-positive MPN. To test the hypothesis that this inherited genetic factor underlies familial clustering in MPN, we performed association analysis of the GGCC haplotype in familial MPN patients. As a result, we could show that the GGCC haplotype confers susceptibility to *JAK2* mutations in familial MPN cases, but does not cause familial clustering. Interestingly, we gained evidence that malignancies other than MPN were more frequent in familial MPN patients who carried the *JAK2* GGCC haplotype. Taken all together, our findings indicate that inherited genetic factors can influence somatic mutability – probably not only in MPN, but as well in other malignant disorders. The exact genetic mechanisms underlying this pathogenetic effect remain yet to be identified.

Abstract in German

Myeloproliferative Neoplasien (MPN) stellen eine heterogene Gruppe maligner myeloischer Erkrankungen dar, denen klonale Hämatopoiese als gemeinsamer pathogenetischer Faktor zu Grunde liegt. Erworbene Gendefekte stehen bekanntlich in Zusammenhang mit abnormer Zellproliferation und klonalem Auswuchs hämatopoietischer Zellen. Die Zielsetzung dieser Arbeit war neue Erkenntnisse über die Rolle rekurrenter genetischer Anomalien in der Pathogenese der MPN zu gewinnen. Deletionen des langen Arms von Chromosom 13 (del13q) wurden in myeloischen Neoplasien bereits beschrieben, wobei ihre genaue Häufigkeit sowie pathogenetische Bedeutung weitgehend unbekannt ist. Mittels hochauflösender Mikromatrix-Genkartierungsanalysen konnten wir insgesamt 16 Patienten mit del13q identifizieren und eine del13q-Häufigkeit von etwa 3% in unserer MPN-Patientenkohorte definieren. Die Zusammenstellung dieser Chromosomenabschnitte lies eine neue minimale deletierte Region von etwa 850 kb erkennen, welche 7 Gene – einschliesslich dem Retinoblastoma 1 (*RB1*) Tumorsuppressorgen – beinhaltete. Genetische und funktionelle Analysen zeigten jedoch keinen pathogenetischen Effekt eines RB1-Allelverlustes in MPN. Daten aus klonalen Analysen hämatopoietischer Vorläuferzellen legten nahe, dass die JAK2-V617F Mutation in einigen Patienten mehr als nur ein Mal erworben wurde. In einer PCR-basierten Untersuchung konnten wir zeigen, dass etwa 3% aller MPN-Patienten die JAK2-V617F Mutation mindestens zweimal erworben hatten. Zudem zeigte sich, dass ein bestimmtes *JAK2*-Genallel anfälliger für V617F als auch Exon 12 Mutationen des *JAK2*-Gens ist. Wir konnten einen Haplotyp identifzieren, der das *JAK2*-Gen beinhaltet und ein höheres Risiko für *JAK2* Mutation-positive MPN birgt. Um zu eruieren, ob dieser vererbbare genetische Faktor der familiären Häufung von MPN zugrunde liegt, führten wir eine Untersuchung familiärer MPN-Fälle durch. Diese zeigte, dass der *JAK2* GGCC Haplotyp auch hier ein höheres Risiko für *JAK2*-Mutationen mit sich bringt, aber nicht die familiäre Häufung in MPN erklärt. Unsere Daten weisen allerdings darauf hin, dass andere maligne Erkrankungen in familiären MPN häufiger mit dem *JAK2* GGCC Haplotyp vergesellschaftet sind. Zusammenfassend zeigt diese Arbeit, dass vererbbare genetische Faktoren die erworbene Mutagenität beeinflussen können. Der genaue genetische Hintergrund dieses pathogenetischen Mechanismus bleibt jedoch aufzuklären.

1 INTRODUCTION

1.1 The myeloproliferative neoplasms

Myeloproliferative neoplasms (MPN) are a heterogeneous group of chronic disorders of the hematopoietic system that are characterized by marked proliferation of the myeloid blood cell lineage. Genetic defects that are acquired within a multipotent hematopoietic stem or very early progenitor cell are thought to initiate excessive proliferation and clonal expansion of myeloid cells. In contrast to other malignant disorders of the hematopoietic tissue, the aberrantly proliferating stem or progenitor cell retains its ability to differentiate in MPN, thus giving rise to mature and mostly functional myeloid peripheral blood cells. Several reports from the nineteenth and early twentieth century note that patients with certain hematologic malignancies often share common clinical features (1–3). However, it was William Dameshek who underscored the overlapping clinical characteristics of these hematopoietic disorders and postulated trilineage myeloproliferation as their underlying cause. In 1951, he attributed chronic myelogenous leukemia, primary myelofibrosis, polycythemia vera, essential thrombocythemia, megakaryocytic leukemia and erythroleukemia (diGuglielmo's syndrome) as a related group of hematopoietic diseases and defined the term „myeloproliferative disorders" as being „*various conditions ...* (that) *are all somewhat variable manifestations of proliferative activity, perhaps due to a hitherto undiscovered stimulus*" (4).

Since Dameshek's definition of the myeloproliferative disorders, many insights have been gained on the pathogenetic factors that cause this group of hematologic malignancies and influence their disease course. In 1960, an abnormally small chromosome, named the „Philadelphia chromosome (Ph)", was identified in patients with chronic myelogenous leukemia (CML) (5). Further investigations revealed that the Philadelphia chromosome arose from a reciprocal translocation between chromosomes 9 and 22, t(9;22)(q34;q11), and caused a fusion of the Abelson murine leukemia virus (*ABL*) gene on chromosome 9 to the breakpoint cluster region (*BCR*) gene on chromosome 22 (6–8). The resulting fusion protein BCR-ABL was soon after demonstrated to hold increased protein tyrosine kinase activity and to induce a

leukemic phenotype (9,10). Thus, the BCR-ABL oncogenic fusion protein was recognized as the disease-causing genetic defect in CML. The identification of a single chromosomal aberration that causes a particular disease phenotype established CML as a pathogenetically distinct entity among the myeloproliferative neoplasms. The subsequent discovery of a drug that inhibits the oncogenic BCR-ABL tyrosine kinase denoted a milestone in the treatment of CML and served as a paradigm of targeted anti-cancer drug development (11). The concept of a single genetic defect that causes a particular phenotype and can be targeted specifically by a drug raised the hypothesis that the other MPN might as well be caused by genetic lesions that can serve as therapeutic targets.

In 2005, a single point mutation in the Janus kinase 2 (*JAK2*) gene was identified in most patients with polycythemia vera and about half of cases with essential thrombocythemia and primary myelofibrosis (12–15). This finding further supported the hypothesis that, as in CML, a unique genetic defect was underlying the pathogenesis of the Ph chromosome negative MPN. However, the *JAK2* mutation was not present in all patients with MPN and clonal hematopoiesis was detected outside the *JAK2* mutation-positive hematopoietic cell clone (16). These findings made clear that the *JAK2* mutation was not the disease-initiating defect and that other genetic factors must be contributing to the pathogenesis of MPN.

In the last years, great effort was taken in order to characterize the genetic defects that impact disease development in myeloproliferative neoplasms. Although several chromosomal aberrations and specific mutations have been identified, the mechanisms by which these genetic alterations promote tumor formation in MPN are not fully understood. Thus, the aim of this study was to gain further insights into the disease biology by characterization of the inherited and acquired genetic factors that contribute to the MPN phenotype and might serve as targets for novel therapeutic approaches.

1.2 Clinial characteristics of the myeloproliferative neoplasms

1.2.1 Polycythemia vera

Polycythemia vera (PV) is characterized by an excessive production of red blood cells and represents the most common primary form of erythrocytosis. PV is a rare disorder with an incidence of about 1.9 to 2.8 per 100,000 person-years (17,18). The most relevant clinical features for the diagnosis of PV are an increased hemoglobin concentration, leukocytosis, thrombocytosis and splenomegaly. Laboratory parameters that aid the diagnosis of PV include bone marrow histopathology, which characteristically shows increased cellularity of the bone marrow with trilineage myeloproliferation and clustering of small to giant polymorphic megakaryocytes (19–21). Further characteristic laboratory findings in PV include high platelet counts, low serum erythropoietin (EPO) levels as well as *in vitro* growth of EPO-independent hematopoietic progenitor cells, which is also referred to as endogeneous erythroid colony (EEC) formation (22–26). Although EECs are not specific for PV and have been observed in other MPN, their presence indicates altered hematopoietic progenitor cell function and thus, excludes secondary causes of erythrocytosis.

Soon after its identification, the *JAK2*-V617F mutation has been implicated in the diagnosis of PV as it is present in more than 95% of patients. Additionally, mutations of exon 12 of the *JAK2* gene have been identified in about 20% of the rare V617F-negative PV cases. Thus, PCR-based assays for the detection of *JAK2* mutations represent useful tools in the differential diagnosis of PV. Chromosomal aberrations such as deletions of chromosome 20q, 13q, trisomies 8 and 9 as well as deletions of 1p have been reported as the most frequent chromosomal abnormalities in PV patients, affecting about 35% of cases (27).

Clinical complications of PV mostly result from increased numbers of circulating peripheral red blood cells, causing high blood viscosity and endothelial dysfunction. Thus, microcirculatory problems such as headache, dizziness, vertigo, flickering sensations of the eyes, as well as pruritus and erythromelalgia are frequent. Thrombotic complications such as cerebrovascular events, acute myocardial infarction, deep vein thrombosis and pulmonary embolism are the most common life-threatening complications and occur in up to 30% of cases (28). The Budd-Chiari

syndrome, representing one of the most fatal complications in PV, results from a thrombotic occlusion of the hepatic veins and can lead to liver ischemia and necrosis, requiring liver transplantation in most severe cases. The “spent phase” of PV represents a serious clinical condition due to the exhaustion of the bone marrow and can lead to terminal transfusion dependency of the patient (29–31). The relative risk of transformation to leukemia, one of the most severe complications of PV, was reported as 6.3 within the first 10 years after PV diagnosis (30,32).

The major therapeutic aims in PV treatment are to ameliorate symptoms and increase the patient’s quality of life. Phlebotomy is recommended for all patients at low risk for thrombosis and those with no or mild microcirculatory problems. Cytoreductive therapy is introduced in cases with disease progression, marked by an increase in spleen size, leukocytosis and thrombocytosis or in cases with increased risk for thrombosis (age older than 60 years, previous history of thrombosis or presence of other cardiovascular risk factors). Cytoreductive therapy includes interferon alpha, which is mostly preferred in patients under the age of 50 years and patients with high platelet counts, and hydroxyurea in cases with marked thrombocytosis and splenomegaly (33). Adjuvant daily low-dose aspirin (100mg per day) is recommended in order to lower the risk of thrombotic complications in PV patients (30).

1.2.2 Essential thrombocythemia

Essential thrombocythemia (ET) is characterized by increased platelet counts in the peripheral blood. The reported prevalence of ET varies between 2.5 to 40 per 100,000 persons (34,35). In most cases, ET will not present with specific symptoms or hemostatic complications. As increased platelet counts can be observed under various clinical conditions such as infections, malignant tumors and in many hematological disorders, the differential diagnosis between reactive and primary forms of thrombocytosis might cause difficulties. Thus, molecular markers such as JAK2-V617F and MPL-W515 mutations serve as essential diagnostic tools in the differentiation between primary and secondary causes of thrombocythemia. The

JAK2-V617F mutation is present in up to 50% of ET patients and is associated with a significantly higher risk for fibrosis, bleeding and thrombosis (14). Somatic gain-of-function mutations at codon 515 of the thrombopoietin receptor (*MPL*) gene are found in about 1% of patients with ET. *MPL* mutations have been demonstrated to alter protein function and induce constitutive activation of the JAK-STAT pathway (36,37). Chromosomal aberrations are relatively rare in ET and affect less than 7% of patients, with trisomy 9 being the most frequent chromosomal abnormality (38).

Bone marrow biopsies of ET patients typically show increased cellularity with megakaryocyte hyperplasia and characteristic clusters of megakaryocytes (39). In contrast to PV, thrombopoietin (TPO) levels are normal or mildly elevated (40). ET patients have an increased tendency to hemorrhages and thrombosis that manifests in various complications from easy bruising and mild microcirculatory problems to microvascular occlusions as well as life-threatening hemorrhagic and thrombotic events that represent the major cause of morbidity and mortality in ET. Of note, very high platelet counts of more than 1,500,000/μL are more often associated with hemorrhagic complications due to acquired von Willebrand disease, whereas platelet counts of less than 1,000,000/μL are mostly associated with thrombotic events (41).

The diagnosis of ET requires exclusion of pathological conditions that might cause secondary thrombocytosis and includes the validation of the JAK2-V617F mutational status as well as other molecular markers such as MPL-W515. Importantly, early stage PV and PMF can initially present with isolated thrombocytosis and thus, have to be critically considered in the differential diagnosis of ET (42–44). Asymptomatic patients with mild to moderately elevated platelet counts will not require therapy, whereas cases with severe thrombocytosis or persistent clinical symptoms should receive cytoreductive treatment with anagrelide – a platelet-specific cytoreductive drug – or hydroxyurea.

1.2.3 Primary myelofibrosis

Primary myelofibrosis (PMF) is characterized by bone marrow fibrosis, myeloid metaplasia with extramedullary hematopoiesis and splenomegaly. Establishing the

diagnosis of PMF can be difficult as there is no disease-specific marker available. Moreover, bone marrow fibrosis with myeloid metaplasia and splenomegaly is frequently observed in other MPN. Fibrotic replacement of the bone marrow tissue in PMF was shown to associate with increased levels of stimulatory cytokines and growth factors such as platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF-ß) that are secreted by activated platelets, megakaryocytes and monocytes (45,46). Excessive levels of stimulatory cytokines in the bone marrow result in increased fibroblast proliferation as well as reticulin and collagen fiber production (47). Although fibroblasts play a central role in the pathogenesis of PMF, they do not belong to the malignant disease clone (48,49).

About one third of PMF patients present with a chromosomal aberrations at diagnosis, of which deletions of chromosomes 13q, 20q and trisomies 8 and 9 as well as partial trisomy 1q are the most frequent (50). Noteworthy, additional chromosomal defects within the MPN clone might be observed during the course of the disease due to the acquisition of additional genetic defects or as a consequence of DNA-damaging cytoreductive therapy. The JAK2-V617F mutation is detected in about 50% of PMF patients, whereas mutations of the MPL gene are found in about 1 to 5% of cases (14,36,37).

Most PMF patients are asymptomatic or present with unspecific symptoms. Blood smears show characteristic teardrop-shaped erythrocytes (dacryocytes) and immature red blood cells as well as myeloid progenitors in the peripheral blood due to altered bone marrow function (51). A characteristic clinical sign of PMF is the dry-tap bone marrow aspirate. Progressive bone marrow fibrosis in advanced PMF promotes extensive extramedullary hematopoiesis in the spleen, liver or other organs and is associated with severe and life threatening complications (52).

The diagnosis of PMF requires other primary and secondary causes of bone marrow fibrosis to be excluded. Clonal molecular markers such as the JAK2-V617F mutation or MPL mutations aid the distinction between PMF and secondary causes of myelofibrosis. However, more than 40% of PMF patients do not present with a chromosomal abnormality and require a thorough diagnostic work-up with special attention to characteristic findings in bone marrow aspirates and biopsies. Overall

survival of PMF patients ranges from 1 to 15 years, with a median survival of about 5 years (53). In severe cases, the clinical course terminates into bone marrow failure with transfusion-dependent anemia and complicated hepatosplenomegaly. An accelerated phase and aggressive form of acute myeloid leukemia, that is mostly non-responsive to therapy, can follow a long chronic disease state.

As there is no curative treatment for PMF, therapy can only be of palliative nature. Splenectomy may be necessary in cases with established hypersplenism and whenever splenomegaly impairs alimentation. Hydroxyurea has been proven useful in controlling organomegaly, whereas interferon alpha, despite many pronounced side effects, might be effective in early stages of the disease in lowering high platelet and white blood cell counts. Glucocorticoids are used in controlling autoimmune complications. In younger patients, allogeneic bone marrow transplantation should be considered as a potentially curative therapeutic option.

1.2.4 Familial MPN

Familial myeloproliferative neoplasms are extremely rare disorders with at least two members of the same pedigree that are affected by PV, ET or PMF. A prevalence of 7.6% was reported for familial cases among apparently sporadic MPN patients (54). Noteworthy, familial MPN patients do not differ from sporadic cases in their clinical presentation, complications, disease course and survival (54,55). The analysis of six pedigrees with familial PV has served evidence that familial MPN follows an autosomal dominant inheritance pattern with incomplete penetrance (56). Linkage analysis of the PV phenotype and a number of chromosomal candidate loci that were implicated in sporadic MPN pathogenesis (chromosomes 9p, 20q, 13q, 5q, the *MPL* and *EPO* gene) revealed no association in familial MPN (52). Thus, the genetic factors that predispose to MPN were suggested to locate outside the investigated chromosomal regions. In further conclusion, alterations in these regions represent secondary genetic events that are acquired throughout the disease course, but do not initiate the disease phenotype.

The identification of the JAK2-V617F mutation in sporadic MPN raised the hypothesis that inherited *JAK2* mutations might underlie familial clustering of MPN. Subsequent analysis of a family with two affected members that were JAK2-V617F positive revealed that the obligate carrier was healthy and, moreover, did not carry the JAK2-V167F mutation (57). Thus, JAK2-V617F was not inherited in this pedigree, but somatically acquired in both affected family members. In familial MPN patients that carried the JAK2-V617F mutation in the granulocytic sample, the mutation was absent in T lymphocytes and thus, could not be of germline origin (54,58,59). Variation in JAK2-V617F mutant allele burden was detected in affected MPN family members, in contrast to a 50:50 ratio between wildtype and V617F mutant alleles as expected for a germline variant, indicating that the mutation was acquired and not inherited. These observations served clear evidence that *JAK2* mutations are acquired and not inherited in familial as well as sporadic MPN (54,58,59). The report of MPN pedigrees with affected family members that display different mutations of the *JAK2* gene such as JAK2-V617F and *JAK2* exon 12 mutations, further supported the notion that a genetic predisposition to the acquisition of *JAK2* mutations is inherited in familial MPN (60). Several studies have shown that common somatic mutations that are involved in the pathogenesis of sporadic MPN – such as mutations in *JAK2*, *MPL* and *TET2* – are not inherited, but somatically acquired in familial MPN cases (54,58,61). So far, the sole exception to this observation was served by the germline *MPL*-S505N mutation, which was identified in a Japanese pedigree with hereditary thrombocythemia (62).

As in sporadic MPN, clonal hematopoiesis represents a hallmark of familial MPN pathogenesis and was shown to precede the acquisition of oncogenic mutations such as JAK2-V617F. Investigations of X-chromosome inactivation in female patients with familial MPN have revealed that clonal hematopoiesis is present in familial MPN cases with a low mutant JAK2-V617F allele burden (54). Furthermore, EPO-independent erythroid progenitor (EEC) growth was not restricted to patients with manifest familial MPN, but was also detected in healthy family members, indicating that the PV stem or disease clone was present in apparently healthy relatives of familial MPN patients (56). These data serve supportive evidence for a sequential acquisition of genetic aberrations such as the JAK2-V617F mutation on the background of clonal hematopoiesis in familial MPN.

The JAK2-V617F mutation is present in up to 78% of cases with familial PV, 69% of ET and close to 90% of familial PMF (54,58). The differences in JAK2-V617F mutation frequencies among familial MPN entities represent one of the major discrepancies between familial and sporadic MPN, which are supposedly due to homogeneous mutation patterns among MPN families. In the majority of pedigrees with familial MPN, somatic mutations such as JAK2-V617F are either present in all or none of the affected members. On the other hand, families with variant mutation patterns have been described as well (54,58,59). Thus, the genetic factors that predispose to familial MPN might differ between families with homogeneous and heterogeneous patterns of acquired oncogenic mutations. The inherited genetic predispositions that initially trigger MPN disease development might co-exist with other germline genetic factors that exert an influence on the acquisition of specific mutations throughout the disease course. In fact, further investigations are necessary in order to elucidate the disease pathogenesis and genetic mechanisms that underlie familial clustering of MPN.

1.3 Molecular pathogenesis of MPN

1.3.1 Clonal hematopoiesis

A major hallmark of the myeloproliferative neoplasms is their common pathogenetic feature of clonal hematopoiesis. In MPN, all myeloid blood cells are derived from a common aberrant stem cell clone, which gives rise to an increased number of peripheral blood cells. The clonal origin of MPN was first demonstrated based on X-chromosome inactivation studies in female CML patients using the glucose-6-phosphate dehydrogenase (G6PD) gene locus. The study of myeloid blood cells from female CML patients that were heterozygous for the G6PD enzyme showed that erythrocytes and granulocytes expressed only one of the two G6PD isotypes (63–66). These data not only suggested a clonal origin of CML, but also raised the hypothesis that erythrocytes and granulocytes originate from the same hematopoietic stem cell clone and that CML was, in fact, a stem cell disorder. Soon after, the same was demonstrated for myeloid blood cells of PV, PMF and ET patients, thus establishing the clonal stem cell origin of these hematopoietic disorders (64,67,68). Further investigations revealed that a varying fraction of B- and T-lymphocytes as well as natural killer cells was clonally involved in MPN, suggesting that in some cases the disease-initiating genetic lesion occurs in an early uncommitted stem cell (58,69–71).

A different approach to investigate clonality in MPN was the application of *in vitro* cultures of hematopoietic progenitor cells that were isolated from the blood or bone marrow of MPN patients. As increased proliferation of myeloid blood cells is the major phenotypic hallmark of MPN, it was hypothesized that altered cytokine responses of hematopoietic progenitor cells might play a role in MPN disease pathogenesis. Accordingly, first results of *in vitro* cultures showed that hematopoietic progenitor cells of MPN patients exhibit hypersensitivity to growth factors and cytokines such as EPO, IGF-1, IL-3 and GM-CSF (72–74). Furthermore, hematopoietic progenitors of PV patients demonstrated growth of erythroid colonies (burst forming unit-erythroid, BFU-E) in complete absence of cytokines, a phenomenon that was referred to as endogenous erythroid colonies (EEC) (26). Cytokine-independent EEC growth was not restricted to PV, but was also detected in PMF and ET patients. Importantly, *in vitro* erythroid colony formation in the absence

of growth factors was never observed in secondary causes of polycythemia and thus, was introduced as a diagnostic marker in MPN. Although the diagnostic importance and implication of *in vitro* EEC growth was limited after the identification of the JAK2-V617F mutation, hematopoietic colony assays still serve as useful scientific tools for the investigation of clonality using X-chromosome inactivation such as the human androgen receptor (HUMARA) assay as well as in defining clonal structures of the hematopoietic progenitor cell pool by PCR-based genotyping methods (75–78).

1.3.2 Altered cytokine signaling

The finding of abnormal responsiveness of hematopoietic progenitor cells to cytokines and growth factors, as observed in MPN patients, raised the hypothesis that alterations in cytokine signaling pathways might underlie the disease phenotype of MPN. Accordingly, scientific investigations in the field of MPN were first focused on cytokine receptors and their ligands. Initial findings suggested a pathogenetic role for mutations of the erythropoietin receptor (EPOR), increased phosphorylation of the insulin growth factor-1 (IGF-1) receptor beta subunit in peripheral blood mononuclear cells of PV patients and decreased expression of the thrombopoietin receptor (MPL) in platelets of MPN patients (79–81). Other observations showed that the activity of the protein thyrosine phosphatase PTP-MEG2 was increased in erythroid colony forming cells of PV patients. PTP-MEG2 was shown to be a membrane-associated phosphate that plays an important role in erythroid cell development. Erythroid progenitor cells of PV patients sustained PTP-MEG2 expression for a comparably longer time period, which was shown to correlate with increased colony-forming capability (82). Furthermore, altered protein kinase C activity and constitutive activation of STAT3 were suggested to cause aberrant erythropoiesis in PV (83,84).

Gene expression analysis in granulocytes of PV patients revealed a novel gene, the polycythemia rubra vera 1 (*PRV-1*) gene, that was highly expressed in granulocytes of PV patients, but was not detected in normal controls or in other myeloid malignancies (85). PRV-1 was identified as a member of the uPAR/CD59/Ly6 family of cell surface receptors that is specifically expressed in

normal human bone marrow, granulocytes of PV patients and to a lesser degree in fetal liver, but not in other tissues (85,86). As PRV-1 expression was not detected in normal granulocytes or myeloid cells of hematopoietic malignancies other than PV, expression analysis of PRV-1 was suggested as a specific diagnostic marker in PV. However, further investigations showed that increased mRNA expression of PRV-1 did not result in higher PRV-1 protein levels at the cell surface (86). Furthermore, G-CSF stimulation of healthy controls was shown to result in granulocytic PRV-1 expression and PRV-1 overexpression was not restricted to PV, but was also present in 50% of ET and PMF patients as well as in patients with hereditary thrombocythemia (85,87,88). These findings led to the conclusion that increased PRV-1 expression in granulocytes of MPN patients might be due to abnormal serum cytokine concentrations and that PRV-1 cannot serve as a PV-specific molecular marker. The identification of the pathogenetic JAK2-V617F mutation and its comparison with PRV-1 expression and EEC growth as molecular markers in the differential diagnosis of PV and secondary erythrocytosis revealed a significant correlation between the JAK2-V617F mutation, overexpression of PRV-1 and the presence of EECs (87,89). However, JAK2-V617F mutation analysis in granulocytic DNA was demonstrated to be superior to PRV-1 expression analysis in diagnostic differentiation of primary and secondary forms of polycythemia (89).

1.3.3 Apoptosis

Another approach to elucidate the molecular mechanisms in MPN was the investigation of apoptotic pathways in hematpoietic cells. The finding of increased expression of the antiapoptotic protein Bcl-x_L in erythroid progenitors of PV patients suggested that myeloid progenitor cells might upregulate Bcl-x_L to prevent apoptosis due to diminishing EPO levels (90). The results of a recent study indicate that alterations of the Bcl-x_L deamination pathway might contribute to the pathogenesis of MPN (91). The Bcl-x_L deamination pathway is activated upon DNA damage and induces apoptosis via inhibition of Bcl-x_L function. In myeloid cells of patients with BCR-ABL positive CML or JAK2-V617F positive PV, DNA damage did not activate this proapoptotic pathway (91). These data suggest that the malignant MPN clone

prevents apoptosis via inactivation of the Bcl-x_L deamination pathway and, in further consequence, accumulates DNA damage over time. Noteworthy, the application of tyrosine kinase inhibitors such as imatinib and JAK2 inhibitors could restore proapoptotic responses in these cells, indicating that aberrant tyrosine kinases might interfere with the regulation of apoptosis in hematopoietic cells.

Further evidences for altered apoptosis pathways in MPN are served by observations such as increased expression of FK506 binding protein 51 (FKBP51), a member of the immunophilin protein family, in megakaryocytes of PMF patients compared to healthy donors (92). FKBP51 has been shown to inhibit calcineurin upon binding to FK506, a selective immunosuppressing drug, and has been suggested to play a role in cell proliferation, differentiation and survival. Overexpression of FKBP51 was demonstrated to cause a decrease in calcineurin phosphatase activity and thus, to inhibit apoptosis in megakaryocytic cells. Interestingly, increased expression of nuclear factor I-B (NFIB) has been observed in granulocytes and $CD34^+$ cells of MPN patients and was shown to cause decreased sensitivity to transforming growth factor ß1 (TGF-ß1), an important negative regulator of hematopoiesis (56,88). TGF-ß had been previously shown to induce fibrotic processes in the bone marrow, to suppress hematopoiesis and increased levels had been found in platelets derived from PMF patients (93–95). However, a direct association between NFIB overexpression and TGF-ß resistance has not been demonstrated yet in cells of PV patients and the significance of this pathway in PV pathogenesis remains to be elucidated. Other apoptosis pathways that have been implicated in PV include the RAS-ERK and phosphoinositide-3 kinase-AKT pathways. A recent study reported that erythroid precursor cells of PV patients exhibit resistance to apoptosis due to increased activity of the RAS and PI3K signaling pathways (96). However, the mechanism of how hyperactivation of these pathways is achieved remains unclear to date.

1.3.4 Cytogenetic aberrations

Significant afford has been taken in order to explain the genetic mechanisms

underlying clonal hematopoiesis in MPN. Cytogenetic analysis revealed a variety of somatically acquired chromosomal abnormalities that are present at diagnosis or occur during the disease course in MPN. The most frequent chromosomal aberrations include acquired uniparental disomy of chromosome 9p, aneuploidy or numerical abnormalities of chromosomes 1, 8 and 9, deletions of chromosomes 5q, 13q and 20q, and gains of 9p (56,97–106). Using a genome-wide microsatellite screening, Kralovics et al. identified acquired uniparental disomy of the short arm of chromosome 9 (9pUPD) in 33% of PV patients, the most frequent chromosomal aberration described in PV at the time (56). Further investigations revealed that 9pUPD was a result of mitotic recombination, which is due to a crossing over between sister chromatids and causes homozygosity for the chromosomal region distal to the crossover (56). Interestingly, 9pUPD does not exclusively occur in PV, but was also detected in PMF and only rarely in ET (14,107). In fact, thorough analysis of the chromosomal region affected by 9pUPD led to the identification of the oncogenic V617F mutation in the JAK2 tyrosine kinase that was demonstrated to play a key role in MPN pathogenesis (14).

According to recent reports, chromosomal aberrations are most frequent in PMF affecting about 35% of patients, whereas up to 20% of PV and less than 7% of ET patients present with cytogenetic abnormalities at the time of diagnosis (50,99,105,108). Of note, karyotypic analysis of erythroid progenitor colonies in *in vitro* cultures has shown that chromosomal alterations are present in only a subgroup of colonies and thus, must have occurred during the course of the disease (109). The consequent accumulation of cytogenetic aberrations over time has been hypothesized to relate to myelosuppressive therapy, which might exert selective advantage to minor therapy-resistant clones that reside in the bone marrow and were previously suppressed by the dominant disease clone. Alternatively, the emergence of distinct subclones might be a result of general genomic instability that is inherent to the disease-initiating clone. In fact, the outgrowth of subclones that harbor multiple and/or distinct chromosomal aberrations within the initial disease clone serves evidence for a sequential acquisition of genetic defects and a “multi-hit” model of clonal evolution in MPN (110). Recent data demonstrate that certain chromosomal aberrations such as deletions of chromosome 7p are detectable in progenitor colonies and that the proliferation and dominance of this particular disease clone

associates with transformation to acute leukemia and a fatal outcome (111).

Deletions of chromosome 20q (del20q) are among the most frequent chromosomal aberrations in MPN. Cytogenetic abnormalities involving chromosome 20q occur not only in MPN, but were also identified in other myeloid malignancies such as myelodysplastic syndrome (MDS) and AML. The observed overlap in the cytogenetic profile among the myeloid neoplasms suggests a common pathogenetic relevance of del20q in these disorders. Cytogenetic analysis using single nucleotide polymorphism (SNP) mapping arrays showed that del20q is present in up to 5% of patients with myeloid neoplasms, being the sole chromosomal aberration in 30% of cases (100). In PV, del20q was previously reported as the most common chromosomal abnormality after 9pUPD with a frequency of 15 to 20% (103,106). The loss of chromosomal material is believed to target tumor suppressor genes within the deleted region and thus, to interfere with protein function and alter cell proliferation and survival in further consequence. Great effort was taken to define chromosomal regions that are deleted in all identified cases and are most likely to harbor the tumor suppressor(s) that are contributing to MPN pathogenesis. Two common deleted regions (CDR) were recently defined for del20q in myeloid malignancies encompassing MPN, MDS and AML: the first CDR spanning a 2.5 Mb region between bands 20q11.23 and 20q12 and the second CDR including a 1.8 Mb chromosomal region within band 20q13.12 (100). Previous cytogenetic studies in PV patients reported a CDR of 2.7 Mb within chromosomal band 20q12 (112). Thorough analysis and characterization of the putative target genes within these common deleted regions is expected to serve further insights into the genetic basis of clonal evolution in MPN.

1.3.5 Deletions of chromosome 13q

Deletions of the long arm of chromosome 13 (del13q) are among the most frequent cytogenetic abnormalities in myeloid as well as lymphoid malignancies (113,114). Recent reports in literature demonstrate that del13q represents the most common chromosomal aberration in B-cell chronic lymphocytic leukemia (B-CLL),

affecting more than 50% of patients (115,116). The frequency of del13q in MPN has been reported to be highest in PMF and post-polycythemic myelofibrosis. About 50% of these patients present with an abnormal karyotype at diagnosis, with del13q being the most common cytogenetic alteration next to del20q, trisomies 8 and 1q (103,105,117,118). As del13q occurs in myeloid as well as lymphoid neoplasms, special attention has been devoted to decipher the specific pathogenetic relevance of these deletions in hematological malignancies. A study of 20 patients with myeloid neoplasms including MPN, CML, MDS and AML showed that the loss of chromosomal material between the bands 13q14 to 13q21 was common to all cases (114). The cytogenetic data of this study delineated a common deleted region of 16 Mb for del13q in myeloid neoplasms.

First karyotype analyses on lymphoid neoplasms led to the identification of chromosomal deletions involving 13q14 as the most common cytogenetic abnormality in B-CLL (119,120). The retinoblastoma tumor suppressor gene (*RB1*), being located at band 13q14, was initially suggested as the potential target of 13q deletions in B-CLL. However, it was soon after proven that the remaining *RB1* allele in del13q positive patients was entirely intact, suggesting a different target in an adjacent locus (113,121). More recent literature on B-CLL reports clustering of the breakpoint of del13q within a 4.1 Mb interval on 13q14. The core-deleted region encompasses 500 kb and exclusively harbors a microRNA cluster encoding the *MIRN15A/MIRN16-1* genes (115). As *RB1* lies outside the reported chromosomal region, these data serve evidence that *RB1* is not the pathogenetically relevant gene of del13q in B-CLL, but suggest the *MIRN15A/MIRN16-1* genes as the likely targets. Moreover, both microRNAs have been shown to be downregulated in B-CLL patients with del13q14 (122). Further data has been provided by a study indicating that deletions of 13q14 are heterogeneous and comprise multiple deletion subtypes, with the *RB1* and *MIRN15A/MIRN16-1* loci representing the anatomical landmarks (116). Supporting evidence for a heterogeneous deletion profile is served by a study using SNP mapping arrays that reveal 2 subgroups of del13q in B-CLL: short/biallelic 13q14 deletions with invariant loss of the *MIRN15A/MIRN16-1* genes versus wide/monoallelic deletions that retain both copies of *MIRN15A/MIRN16-1* and thus, do not target the microRNA cluster. Furthermore, expression of *MIRN15A/MIRN16-1* was only significantly reduced in cases with biallelic deletions (123).

Deletions of chromosome 13q have been also observed in hematological malignancies such as multiple myeloma and other lymphoid neoplasms (124,125). The frequent occurrence of del13q in myeloid as well as lymphoid malignancies suggests a hematopoietic lineage-specific function of 13q alterations. This hypothesis is supported by the observation that the prognostic impact of del13q differs between disease entities. In CLL, del13q is associated with a better prognostic outcome, whereas patients with multiple myeloma that carry 13q deletions have a poor prognosis (124,126). Clinical data of MPN patients positive for del13q show that 13q deletions as sole chromosomal abnormalities do neither influence survival nor correlate with other clinical features such as transformation to leukemia (127).

Deletions of chromosome 13q are well studied in lymphoid leukemia due to their high frequency and obvious relevance for disease pathogenesis. On the contrary, the function and significance of 13q deletions is not well characterized in MPN. Available literature on del13q in MPN reports a CDR of about 16 Mb located between bands 13q14 and 13q21, invariably including the *RB1* gene locus. However, cytogenetic data on chromosome 13q deletions in MPN are only limited and the functional impact of *RB1* loss on MPN disease pathogenesis is yet unclear. Del13q represents one of the most common chromosomal aberrations in PMF and PV. Thus, it is of major interest to elucidate the role of this recurrent cytogenetic abnormality in order to shed light to the genetic mechanisms that underlie clonal evolution in MPN.

1.3.6 The JAK2-V617F mutation

In 2005, four independent investigations resulted in the identification of an acquired mutation in the Janus kinase 2 (*JAK2*) gene in patients with MPN (12–15). Based on the previous observation that one third of PV patients displayed loss of heterozygosity on the short arm of chromosome 9, DNA sequencing of candidate genes in that specific chromosomal region was performed and led to the identification of a guanine to thymine transversion in exon 14 of *JAK2*, causing a phenylalanine to valine substitution at position 617 (JAK2-V617F) (14).

The Janus kinases (JAKs) represent protein tyrosine kinases that are

constitutively associated with cytokine receptors that do not hold intrinsic kinase activity such as EPOR, MPL and GCSFR. The V617F mutation was shown to alter the catalytically inactive pseudokinase domain (JH2) of JAK2, which serves autoinhibitory function (128,129). The loss of the JH2 domain function establishes constitutive activation of the JAK2 tyrosine kinase and results in increased activation of multiple downstream signaling targets such as the signal transducer and activator of transcription (STAT) family, mitogen activated protein kinase (MAPK) and phosphoinositol 3-kinase (PI3K)-Akt pathways. Signaling through these pathways leads to activation of genes that are implicated in hematopoietic cell proliferation and survival. Thus, increased JAK2 signaling activity grants growth and selective advantage to the cell that acquired the JAK2-V617F mutation and establishes clonal hematopoiesis (130).

The reported frequencies of JAK2-V617F are about 95% in PV patients, whereas 50% of ET and PMF patients are affected (131). Although it is present in the majority of MPN patients, the JAK2-V617F mutation is not specific for MPN, but has also been found with lower frequencies in patients with other myeloid neoplasms such as CML, MDS or AML (132–135). Activation of the JAK-STAT signaling pathway is generally a common oncogenic event that has been reported in a variety of malignant disorders (136). Conversely, the JAK2-V617F mutation itself has neither been detected in solid tumors nor in hematopoietic malignancies of the lymphoid lineage (136).

First investigations in MPN using hematopoietic progenitor cell cultures revealed that myeloid progenitors of MPN patients exhibit hypersensitivity to several cytokines such as EPO, IL-3, GM-CSF, and IGF-1 (72–74). Moreover, spontaneous growth of myeloid progenitor colonies in the absence of cytokines and growth factors was observed in patients with primary, but not secondary forms of polycythemia (26). After the identification of the JAK2-V617F mutation, functional analyses of the mutant JAK2 kinase revealed that it establishes hypersensitivity to hematopoietic cytokines and induces cytokine-independent growth of myeloid cells and thus, underlies these MPN-specific features (12–15). Various *in vivo* studies of mouse models served further insights into the function and impact of JAK2-V617F on MPN pathogenesis. First results of murine bone marrow transplantation experiments revealed that recipient mice of JAK2-V617F transduced bone marrow cells develop severe

erythrocytosis, indicating that the JAK2-V617F mutation promotes increased erythroid proliferation (13). The transplantation of primary hematopoietic cells that express either wild-type JAK2 or JAK2-V617F into lethally irradiated recipient mice was shown to induce a phenotype similar to human PV and myelofibrosis including high hematocrit, leukocytosis, megakaryocyte hyperplasia, bone marrow fibrosis, extramedullary hematopoiesis and splenomegaly (137). Similar results were obtained in an independent study demonstrating that mice reconstituted with JAK2-V617F transduced bone marrow cells developed polycythemia, macrocytosis and granulocytosis, followed by phenotypic features of myelofibrosis after 3 to 4 months post-transplantation (138). These results indicate that the JAK2-V617F mutation is sufficient to induce polycythemia and promotes disease progression to myelofibrosis.

Several lines of evidence indicate that the JAK2-V617F mutation is acquired in a multipotent lympho-myeloid progenitor cell. First, JAK2-V617F was shown to be present in $CD34^+$, $CD38^-$, $CD90^+$ and lineage negative hematopoietic stem cells as well as their progeny and was demonstrated to propagate increased erythroid differentiation (139). Further studies revealed that JAK2-V617F was also present in B-cells and natural killer cells in some rare cases of PV and about 50% of patients with PMF (140). In a small fraction of PMF patients, the mutation was also detectable in T-cells (140). Murine bone marrow transplantation experiments have demonstrated that $CD34^+$ hematopoietic stem cells isolated from JAK2-V617F positive PV or PMF patients are capable of repopulating lethally irradiated immunodeficient mice (141). The recent observation that endothelial cells of MPN patients were positive for JAK2-V617F raised the hypothesis that the mutation was acquired in a common endothelial-hematopoietic progenitor cell (142,143).

Since the identification of the JAK2-V617F mutation, many insights have been gained on MPN disease pathogenesis. However, the mechanism of how a single mutation can induce several distinct phenotypes remains unknown. Data from transgenic mouse models suggest that the expression ratio between wild-type and mutant JAK2 determines the MPN phenotype (144). This hypothesis was supported by the observation that patients with high JAK2-V617F mutant allele burden due to homozygosity for the mutation (caused by mitotic recombination) exhibit a PV phenotype, whereas ET patients usually carry monoallelic JAK2 mutations and thus, have a low V617F allele burden (145). Other findings indicate a role for the genetic

background in the determination of a JAK2-V617F associated disease phenotype. Primary bone marrow cells transduced with JAK2-V617F and transplanted into lethally irradiated Balb/c and C57Bl/6 mice induce a PV-like phenotype with strain-specific differences: whereas recipient mice of the Balb/c strain show marked leukocytosis, splenomegaly and bone marrow fibrosis, C57Bl/6 recipient mice did not show these phenotypic features (137). A subsequent study of various single nucleotide polymorphisms (SNP) in candidate genes such as *EPOR*, *MPL*, *GCSFR* and *JAK2* revealed that certain SNP variants of the *JAK2* and *EPOR* genes are associated with a PV phenotype, but not with ET or PMF (146). Still, further investigations are necessary in order to determine the contribution of genetic variation to the manifestation of a particular MPN disease entity and to decipher phenotypic variability in MPN.

Soon after its identification and characterization, the JAK2-V617F mutation was implemented into the diagnostic criteria and classification scheme of the MPN (147). The mutation is present in virtually all patients with PV and about half of patients with ET and PMF, and thus serves as an important pathogenetic marker in the differential diagnosis of primary and secondary forms of myeloid cytosis. Furthermore, it represents a potential therapeutic target due to the fact that many MPN-specific clinical features relate to the JAK2-V617F. Currently, several trials are evaluating the potential of JAK2 kinase inhibitors in the treatment of hematopoietic neoplasms. Preliminary results indicate that although JAK2 inhibitor therapy does not eradicate the malignant disease clone, it is beneficial for patients in alleviating constitutional symptoms and especially in reducing splenomegaly (148). Further investigations will reveal the actual therapeutic value of JAK2 inhibitors and will grant insights into pathogenetic mechanisms of aberrant JAK2 kinase activity in MPN.

1.3.7 Other oncogenic mutations

After the discovery of the JAK2-V617F mutation and the observation that a significant proportion of MPN patients do not carry the JAK2-V617F mutation, further effort was taken in order to identify novel mutations of the *JAK2* gene as well as in

other candidate genes of the JAK-STAT signaling pathway. A screening of patients with JAK2-V617F negative PV revealed the presence of somatic gain-of-function mutations in exon 12 of the *JAK2* gene in up to 80% of cases (60,70,149). Interestingly, PV patients with *JAK2* exon 12 mutations exhibited a distinct phenotype with isolated erythrocytosis and erythroid hyperplasia of the bone marrow without involvement of the megakaryocytic or granulocytic lineage (149). Moreover, *JAK2* exon 12 mutations were not found in ET or PMF patients, thus suggesting that exon 12 mutations of *JAK2* associate with a distinct clinical phenotype and represent a specific subtype of PV (149).

In a recent investigation, approximately 20,000 DNA samples of patients with apparent MPN were sequenced and led to the identification of various rare mutations within exons 12 to 15 of the *JAK2* gene. Most of the mutations were accordingly located inside the JH2 domain and are thus likely to alter JAK2 function in a similar fashion as the known JAK2-V617F and exon 12 mutations (150). The same investigator group showed in a different experimental approach that about one third of patients with confirmed or suspected MPN carry a deletion of *JAK2* exon 14 (*JAK2* Deltaexon 14). This intragenic deletion was demonstrated to result in expression of a truncated JAK2 protein (151). The functional impact of these novel *JAK2* aberrations in MPN pathogenesis remains to be elucidated.

After the finding of mutations in the *JAK2* gene, further efforts were taken in order to identify novel pathogenetic mutations in genes that encode important cytokine signaling molecules in myeloid cells. Consequently, exon sequencing of the *EPOR*, *MPL* and *GCSFR* genes revealed a novel somatic activating mutation in exon 10 of the *MPL* gene (37). The mutation was shown to result in a tryptophan to leucin substitution (MPL-W515L) in the transmembrane domain of the TPO receptor and to establish constitutive JAK-STAT signaling as well as TPO hypersensitivity in MPL-W515L transfected cell lines. Further *in vivo* data from murine bone marrow transplant experiments showed that mice reconstituted with MPL-W515L transduced primary cells developed an MPN-like phenotype with marked thrombocytosis, splenomegaly and marrow fibrosis (37). Screenings of patients with myeloid malignancies led to the identification of an additional *MPL* mutation, MPL-W515K, and revealed an overall frequency of MPL-W515 mutations of up to 5% in PMF and 1% in ET (36). Interestingly, no mutations of the *MPL* gene were detected in PV

patients or other myeloid neoplasms, suggesting a specific role for *MPL* mutations in PMF and ET pathogenesis. Of the 850 MPN patients involved in the study, six cases that were positive for both, MPL-W515L and JAK2-V617F, were identified. Thus, *JAK2* and *MPL* mutations are not mutually exclusive but co-occur in a small fraction of MPN patients (36).

Recently, somatic loss-of-function mutations of the SH2B adaptor protein 3 gene (*SH2B3*, also known as *LNK*) were identified in two patients with MPN (152). LNK is a member of the adaptor family of proteins and a key negative regulator of hematopoietic cytokine signaling. Among other functions, LNK has been shown to inhibit phosphorylation of the JAK2 kinase as well as the mutant JAK2-V617F (153). The pleckstrin homology (PH) domain of LNK was previously shown to mediate colocalization of LNK to the cell membrane, whereas protein binding to the erythropoietin receptor, the thrombopoietin receptor and the JAK2 kinase is achieved through the Src homology 2 (SH2) domain (154–156). Lnk-deficient mice have been demonstrated to exhibit phenotypic features of human MPN (157,158). Taken together, these data indicate that LNK is an essential negative regulator of JAK-STAT signaling upon activation of EPO and TPO receptors and implicate that the loss of LNK function might contribute to MPN pathogenesis. Accordingly, sequence analysis of 33 JAK2-V617F negative MPN patients revealed two cases with LNK mutations (152). A PMF patient carried a 5-bp deletion with a missense mutation that resulted in an abrogation of both, the PH domain and the SH2 domain of LNK. A second missense mutation leading to a glutamic acid to glutamine substitution (E208Q) in the PH domain of LNK was detected in an ET patient (152). Further sequence analysis of the *LNK* gene in MPN patients resulted in (i) the identification of additional mutations that occur mostly in exon 2 of *LNK*, (ii) demonstrated an 3 to 6% frequency of LNK mutations in MPN, (iii) indicated that these mutations are mostly associated with blast-phase MPN and (iiii) showed that LNK mutations as well as mutations like JAK2-V617F and MPL-W515L are not mutually exclusive but co-occur only rarely due to their low overall frequency (159).

Apart from somatic mutations that result in a deregulation of the JAK-STAT signaling pathway, mutations in genes with epigenetic regulatory function have been identified in MPN. A thorough molecular analysis of 320 patients with myeloid neoplasms (MDS, MPN and AML) disclosed various mutations in the tet oncogene

family member 2 (*TET2*) gene in about 15% of cases (160). An additional screening in a number of patient cohorts with various myeloid malignancies revealed that *TET2* mutations occur in all coding regions of the gene and that they are found in all entities among the myeloid neoplasms. The reported frequencies of *TET2* mutations in overall MPN vary between 8 to 13%, being highest in PV (19%) and lowest in ET (5%) (61,153,161). *TET2* encodes a methylcytosine deoxygenase that has been shown to oxidize the 5-methyl group of cytosine and to generate 5-hydroxymethylcytosine, which represents an intermediate step of DNA demethylation (162,163). A recent study demonstrated that AML patients with *TET2* mutations exhibit a hypermethylation phenotype with about 129 differentially methylated regions over the entire genome that were hypermethylated (164). On the contrary, an independent report has provided evidence that there is no relationship between low levels of 5-hydroxymethylcytosine and DNA hypermethylation (165). Thus, the exact mechanisms by which alterations of the methylcytosine deoxygenase TET2 induce or contribute to the development of myeloid malignancies need to be further studied.

Massively parallel DNA sequencing of a bone marrow and a skin-biopsy sample from a patient with AML, who presented with a normal karyotype at diagnosis, revealed 12 somatic mutations within the genomic coding region and led to the identification of a mutation in the isocitrate dehydrogenase 1 (*IDH1*) gene. A subsequent screening of additional 187 AML cases showed that mutations causing a substitution of the arginine residue at codon 132 of *IDH1* were present in 8% of AML patients and, interestingly, in 16% of samples that were cytogenetically normal (166). The evaluation of mutations in *IDH1* and its homologue *IDH2* demonstrated that (i) *IDH1* mutations occur exclusively at position R132, whereas *IDH2* mutations affect positions R140 and R172; (ii) the frequency of *IDH1/2* mutations is only 1 to 5% in chronic phase MPN, but as high as 21.6% in blast-phase MPN, and (iii) the presence of *IDH1/2* mutations in blast-phase MPN predicts a worse prognostic outcome (167). IDH1 and IDH2 are citric acid cycle enzymes that catalyze the oxidative carboxylation of isocitrate to 2-oxoglutarate and serve an important role in cytoplasmic NADPH production. Mutations in IDH1 have been shown to establish novel enzymatic activity resulting in the production of 2-hydroxyglutarate (168). Thus, it has been hypothesized that *IDH1/2* mutations cause a shift from the production of 2-oxoglutarate to increased 2-hexoglutarate generation. As a result, less substrate is

available for 2-oxoglutarate dependent enzymes such as TET2. Indeed, further studies showed that *IDH1/2* mutations and *TET2* mutations are mutually exclusive, that mutations of *IDH1/2* are associated with an increase in DNA methylation and that mutant forms of the IDH proteins inhibit hydroxylation of 5-methycytosine via TET2 (164).

Approaches through SNP mapping arrays and candidate gene sequencing in MDS and MPN patients recently revealed somatic mutations of the enhancer of zest homolog 2 (*EZH2*) gene on chromosome 7 (100,169,170). Various *EZH2* mutations throughout the gene were identified and shown to associate with acquired UPD at chromosome 7q, resulting in homozygosity for the mutation and a poor prognosis compared to patients without *EZH2* mutations. Cases with a heterozygous mutation state were also reported. Moreover, heterozygosity for EZH2 mutations was associated with a by trend better prognostic outcome compared to patients with homozygous mutations. In MPN, EZH2 mutations were predominantly detected in PMF patients with the highest reported frequency being up to 13% (169). *EZH2* encodes a highly conserved histone H3 lysine 27 (H3K27) methyltransferase and a member of the polycomb repressive complex 2 (PRC2) that has been implicated in epigenetic regulation of embryonic development and stem cell differentiation. Accordingly, mutations in EZH2 have been shown to result in a loss of H3K27 trimethylation in cell lines expressing mutant forms of EZH2 (169).

Mutations of the additional sex combs 1 (*ASXL1*) gene were identified using array comparative genomic hybridization and candidate gene sequencing in about 8% of MPN patients and more frequently in other myeloid neoplasms (171). Reports of other frequently mutated genes that have been implicated in MPN pathogenesis include *CBL*, *RUNX1*, *IKZF1*, and *TP53*. Some of the identified gene mutations occur predominantly in chronic phase MPN (*MPL*, *TET2*, *CBL*), whereas other mutations have been shown to associate with disease progression and transformation to leukemia (*IDH1/2*, *ASXL1*, *IKZF1* and *TP53*) (111,172–174). Still, the exact mechanisms of how mutations in these genes impair hematopoietic cell function and promote a specific disease phenotype remain mostly unclear.

2 AIMS

Throughout the last years, a variety of different chromosomal aberrations and mutations have been identified in patients with MPN, indicating that alterations of different pathways contribute to MPN disease pathogenesis. Still, a major proportion of patients does not carry any of the known aberrations, but is likely to harbor a yet unknown genetic defect. In this study, we aimed to gain further insights into the genetic mechanisms that cause and modulate the disease phenotype in MPN via the application of currently available genomic techniques. We assembled a unique single-institutional cohort of MPN patients and performed cytogenetic analysis in order to identify somatic aberrations that are present at diagnosis or are acquired during the course of the disease. We aimed to further characterize the detected genetic defects that were identified in the initial screening and to define their pathogenetic role in MPN disease evolution.

Early in our investigations we gained evidence that deletions of chromosome 13q occur frequently in MPN patients. Although 13q deletions have been described in literature in various myeloid malignancies, only limited cytogenetic and practically no functional data is available in MPN up to date. Previous studies revealed that the tumor suppressor gene *RB1* is located inside the known common deleted region and was proposed as the putative target gene of del13q. Thus, we aimed to characterize del13q in MPN patients with the specific aims of (i) determining the frequency of 13q deletions in MPN patients, (ii) defining the common deleted region of del13q in MPN and (iii) performing functional analysis in order to identify the target genes of del13q and elucidate their impact on MPN disease pathogenesis. The results obtained will serve insights into the genetic mechanisms of disease development and can aid to improve classification schemes, risk stratification and options for therapeutic intervention in MPN. As deletions of chromosome 13q are found in other myeloid as well as lymphoid malignancies, the characterization of del13q will contribute to the knowledge of genetic mechanisms in a variety of hematologic malignancies.

3 METHODS

3.1 General methods

3.1.1 Patient cohorts and DNA isolation

A total number of 426 patients from Vienna, Austria and 79 patients from Florence, Italy, were included in this study, which was approved by the respective local ethics committees. Peripheral blood samples were collected after written informed consent was obtained from the patients. Granulocytes and mononuclear cells were isolated from peripheral blood using density gradient centrifugation. Genomic DNA from peripheral blood granulocytes and mononuclear cells was isolated using standard protocols.

3.1.2 Allele-specific PCR for the detection of JAK2-V617F

Allele-specific PCR (AS-PCR) assays consist of one common unlabeled and two fluorescent-labeled primers. The AS-PCR assay for the detection and quantification of the *JAK2*-V617F mutation used in this study contains three primers: one common unlabeled sense primer and two 5' fluorescence-labeled anti-sense primers, one of which is specifically binding to the wild-type and the other to the G1849T mutant allele of *JAK2* (Table 1). The mutant-specific PCR primer is extended by 3 non-homologous nucleotides that allow the discrimination of the PCR products by size. The JAK2-V617F mutational load (in %T) can thus be determined using the peak fluorescence ratio between wild-type (G) and mutant (T) PCR products according to the following formula:

%T allele = (T peak height) / (sum of T and G peak heights) x 100

PCR reactions containing granulocyte genomic DNA were prepared according to Table 2 and PCR was performed using an initial polymerase activation step at 95°C for 5 minutes, followed by 35 cycles with denaturation at 94°C for 30 seconds, annealing at 62.2°C for 30 seconds and extension at 72°C for 30 seconds, as well as a final extension step at 72°C for 15 minutes and hold at 8°C. The obtained PCR products were processed through capillary electrophoresis with the Genetic Analyzer 3130xl (Applied Biosystems) according to the manufacturer's guidelines and the data was analyzed using the GeneMapper software (Applied Biosystems).

3.2 Deletions of chromosome 13q in MPN

3.2.1 Microsatellite PCR

Microsatellite PCR was conducted in order to screen the patient cohort for cases with a loss of heterozygosity (LOH) on the long arm of chromosome 13. For each patient, microsatellite PCR was performed on genomic DNA of both, peripheral blood granulocytes (monoclonal tumor tissue) and peripheral blood mononuclear cells (polyclonal control tissue). PCR reactions were prepared according to Table 2 and PCR was performed with an initial polymerase activation step (95°C for 5 minutes), 10 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds, followed by 26 additional amplification cycles with denaturation at 89°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds as well as a final extension step at 72°C for 20 minutes and hold at 8°C. Subsequently, the obtained PCR products were processed through capillary electrophoresis using the Genetic Analyzer 3130xl (Applied Biosystems) and data was analyzed with the GeneMapper software (Applied Biosystems). Only cases with heterozygosity for the respective microsatellite marker were used for LOH analysis. LOH was scored positive if the individual was heterozygous for the microsatellite marker in the mononuclear cell DNA (control tissue), but displayed homozygosity for the same marker in the granulocyte DNA (tumor tissue) sample. Full LOH was scored in cases were one allele of the microsatellite marker was completely lost, whereas partial LOH was suspected in

cases with a difference in allelic ratios between the granulocyte and mononuclear cell DNA samples. Three independent PCR analyses were performed for all microsatellite markers in all cases with a suspected LOH and only samples with a significant difference in allelic ratios between the granulocyte and mononuclear cell DNA were scored as LOH positive (unpaired t-test, $p<0.5$). Table 3 lists the microsatellite markers and primer sequences used in this study.

3.2.2 TaqMan SNP genotyping

In order to increase informativity of the LOH analysis in the MPN patient cohort, two single nucleotide polymorphisms (SNP) were added to the screening, one of which was located inside the *RB1* gene region (*rs9535032*, TaqMan SNP genotyping assay number C__11181793_10) and the another downstream of the microRNA 15a/16-1 cluster region (*rs9535416*, TaqMan SNP genotyping assay number C__27831668_10) on chromosome 13q. Screening of patients for LOH at these loci was conducted by comparison of the SNP alleles between the granulocytes DNA sample (clonal myeloid cells) and buccal mucosa or T-cell DNA samples (polyclonal control sample). Accordingly, only patients that were heterozygous for the respective SNP were informative for LOH analysis. The PCR reaction and genotyping analysis was performed according to the manufacturer's recommendations using the 7900HT Real Time PCR system (Applied Biosystems).

3.2.3 Microarray mapping analysis

Genomic mapping analysis applying microarray technologies was used in order to confirm the cases of LOH at chromosome 13q that were identified in our LOH screening analysis with microsatellite and SNP PCR assays. Genomic DNA samples from MPN patients with suspected 13qLOH (peripheral blood granulocyte or peripheral blood mononuclear cell genomic DNA) were processed for microarray genotyping using the Genome-Wide Human SNP 6.0 mapping arrays (Affymetrix)

according to the manufacturer's guidelines. Subsequently, analysis of copy number and LOH was conducted using the Genotyping Console software version 3.0.2 (Affymetrix).

3.2.4 Colony assays

Peripheral blood mononuclear cells were isolated using density gradient centrifugation according to standard protocols. The isolated mononuclear cell fraction was plated in H4431 methylcellulose media (StemCell Technologies) at a density of 10^5 cells/ml and cultured for 14 days at 37°C. Subsequently, single burst forming units-erythroid (BFU-E) and colony forming units granulocytic-monocytic (CFU-GM) were picked into 5% of Chelex-100 resin solution (Biorad) in 5 mM TRIS pH 8, 0.5% Triton-X100. The single colony samples were incubated for 15 minutes at 56°C and boiled at 96°C for 8 minutes. One microliter of lysate was used for PCR based genotyping. Each of the isolated DNA samples from single colony forming units (CFU) was assayed for the presence of the JAK2-V617F mutation as well as a loss of heterozygosity on chromosomes 13q and 20q. Positivity for the JAK2-V617F mutation was assessed by AS-PCR as described in Methods 3.1.2. LOH on chromosome 13q was determined using microsatellite PCR as defined in Methods 3.2.1. Accordingly, LOH on chromosome 20q was detected by microsatellite PCR using the microsatellite marker *D20S96*.

3.2.5 Gene expression analysis of del13q CDR genes

Peripheral blood granulocytes from MPN patients were isolated using gradient centrifugation according to standard procedures. Cord blood was obtained from five healthy donors and processed through gradient centrifugation. The obtained mononuclear cells were sorted for CD34 expression by magnetic cell sorting (Miltenyi Biotech) in order to obtain the CD34+ and CD34- mononuclear cell fractions. For murine gene expression analysis, bone marrow cells were isolated from the femur

and tibia of three C57/Bl6 mice and magnetically sorted into lineage marker positive and negative fractions using the Lineage Cell Depletion Kit (Miltenyi Biotech). Real time PCR was performed with the 7900HT Real Time PCR system (Applied Biosystems) according to the manufacturer's recommendations. Table 4 lists the TaqMan gene expression assays that were available for this study, including the human and murine HPRT1 assays used for normalization of the obtained gene expression data. The Nudt15 gene expression assay was not available at the time of analysis. However, public database research showed that NUDT15 is expressed in CD34+ hematopoietic cells as well as in several leukemia cell lines (data not shown).

3.2.6 Sequence analysis

Sequence analysis of the *RB1* gene was performed on granulocyte genomic DNA of 4 patients with del13q. All 27 coding exons of the *RB1* gene were sequenced using the primer sequences as listed in Table 5. Exon PCR amplification was performed with 1 ng of DNA and 5 Units of AmpliTaq Gold polymerase in Gold buffer and 1.5 mM magnesium chloride (Applied Biosystems). PCR reaction was performed using the 2720 Thermal Cycler (Applied Biosystems) with an initial denaturation step at 95°C for 10 minutes, followed by 10 cycles of 94°C for 30 seconds, annealing at 65°C for 30 seconds with decreasing temperatures of 1°C per cycle, and 72°C for 30 seconds. Then, 26 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds as well as a 10 minute final extension at 72°C were performed. The sequencing reaction was completed using the BigDye Terminator v3.1 Cycle Sequencing chemistry and the 3130xl Genetic Analyzer (Applied Biosystems) according to the manufacturers recommendations. Data analysis of the obtained sequences was conducted using the Sequencher software version 4.9 (Gene Codes).

3.2.7 Next generation sequencing

First, whole genome amplification of granulocyte genomic DNA from a patient with del13q (P10) was performed using the REPLI-g UltraFast kit (Qiagen) according to standard procedures. The amplified DNA was next processed through DNA fragmentation, adaptor ligation, cluster generation and synthesis sequencing using the Illumina HiSeq 2000 sequencing system according to the manufacturer's guidelines (Illumina). The obtained whole genome sequence reads were aligned to the NCBI36/hg18 reference genome using the CASAVA software (Illumina). The coverage of the reads over the template sequence was determined as number of reads per base. Figure 4A depicts the sequence coverage of each base of chromosome 13q in sample P10.

3.2.8 Del13q breakpoint PCR

In order to determine the breakpoints of the chromosome 13q deletion of patient P10, three PCR primer pairs covering the suspected breakpoint region were designed. Each of these primer pairs consisted of one sense primer upstream of the centromeric breakpoint and an antisense primer located downstream of the telomeric breakpoint of the deletion. Primer pairs were designed to yield PCR products of about 500 to 600 base pairs length if the corresponding sense and antisense primer regions were in close adjacency due to the deletion. PCR products were not obtained for the undeleted allele, as a chromosomal distance of more than 3 mega base pairs between the primer pairs was not supported by the used PCR amplification chemistry. Using three primer pairs, a total of nine distinct PCR amplifications of the del13q breakpoint region were performed as described in Methods 3.2.6. The obtained sequence reads were aligned to the reference sequence NCBI36/hg18 using the UCSC Genome Browser website (http://genome.ucsc.edu). Table 6 lists the primer sequences of the del13q breakpoint PCR of patient P10.

3.2.9 Methylation-specific PCR

Granulocyte genomic DNA was first bisulfate treated for conversion of cytosine residues into uracil. Of note, methylated cytosine residues (5-methylcytosine) remain unaffected by bisulfate treatment. For methylation-specific PCR, the amplification reaction was set up with one microliter bisulfite converted DNA in a final volume of 25 microliters. PCR amplification was performed under the following conditions: 95°C for 10 minutes, 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds, followed by a final step of 72°C for 30 minutes. This PCR reaction yielded methylated (maternal) and unmethylated (paternal) PCR products of 126 bp and 119 bp length, respectively. The obtained PCR products were processed through capillary sequencing using the 3100 Genetic Analyzer and data was analyzed using the Genescan software (Applied Biosystems). For normalization, the ratio of the fluorescence peak areas of methylated and unmethylated PCR products was normalized to the mean ratio of four normal blood samples known to exhibit approximately 50% of methylation. The PCR primers used for methylation-specific PCR are shown in Table 7.

3.2.10 RNAi interference and XTT proliferation assays

We designed shRNAs in the lentiviral pLKO.2 vector (kindly provided by Sebastian Nijman) targeting RB1 and transfected UT7/TPO cells (a thrombopoietin-dependent human megakaryocytic cell line), BaF3/EPO cells (a EPO-dependent murine pro-B-cell line) and murine primary bone marrow cells to knock-down RB1 expression. UT7/TPO and Baf3/EPO cells were cultured in RPMI-1640 medium (Invitrogen) that was supplemented with 10% FCS (Invitrogen) and the essential cytokines TPO and EPO, respectively (TPO: 20% of CM cell line supernatant, EPO: 1 U/ml recombinant erythropoietin ERYPO, Janssen-Cilag). Viral packaging, preparation of viral supernatant and viral transfection was performed as described elsewhere (111,175,176). Primary bone marrow cells were isolated from tibia and femurs of C57BL/6 mice and cultured in StemPro-34 serum free medium (Invitrogen) with nutrient supplement containing each 10ng/ml of recombinant SCF, Flt3-L, GM-

CSF, IL-3 and IL-6 (R&D Systems). Adherent cells were depleted by an incubation period of 24 hours at a density of 10^6 cells/ml and followed by magnetic cell sorting using the Lineage Cell Depletion Kit (CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and CD235a; Miltenyi Biotech). Viral transduction was achieved by incubation of lineage negative mouse bone marrow cells with viral supernatant and supplemented media for 20 hours, followed by a puromycin-selection (2.5 mg/ml) for 4 days.

The knock-down efficiency was confirmed with standard Western blotting in case of UT7/TPO cells and with Rb1 gene expression analysis in case of BaF3/EPO and primary bone marrow cells (data not shown). The following TaqMan gene expression assays were used: for human constructs assays RB1 Hs01078066_m1 and HPRT1 Hs99999909_m1, and for murine constructs assays Rb1 Mm00485586_m1 and Hprt1 Mm01545399_m1, respectively (Applied Biosystems). For functional assessment, XTT assays based on a yellow to orange formazan dye conversion were performed. This conversion only occurs in metabolically active cells and is measured using a standard spectrophotometric reader. Rb1 shRNA transfected cells and control cells transfected with a random shRNA were plated into 96-wells (2500 to 5000 cells per well in triplicates) and cultured under equal conditions with medium containing increasing cytokine concentrations. After 4 to 5 days, cells were stained with XTT solution using the Cell Proliferation Kit II XTT (Roche) and the dye concentration, resembling the amount of viable cells per well, was measured. Proliferation curves were created to visualize the growth pattern and cytokine sensitivity of cells transfected with either Rb1-targeting vector or the control shRNA. The shRNA constructs used in this study are listed in Table 8.

3.3 The *JAK2* haplotype

3.3.1 Patient characteristics

Collection of blood samples was performed at the Medical University of Vienna and was approved by the local ethics committee. Written informed consent was obtained from patients in accordance with the Declaration of Helsinki of 1975, as revised in 2000. A total number of 333 MPN patients and 99 demographically matched non-MPN cases (control population) were included into the study. The diagnosis of MPN was established in accordance with the WHO 2008 criteria (147,177,178).

3.3.2 DNA samples and genotyping

Peripheral blood samples were fractionated into mononuclear cells and granulocytes using density gradient centrifugation. Granulocyte genomic DNA was used for determination of JAK2-V617F mutational status and for analysis of uniparental disomy as described in Methods 3.1.2. For SNP genotyping, DNA of non-myeloid cells (buccal cells or T cells) was used to eliminate false homozygosity calls due to uniparental disomy on chromosome 9p. SNP genotyping was performed with commercially available TaqMan assays (Applied Biosystems) and analyzed on the 7900HT Real Time PCR system according to the manufacturer's recommendations. The following TaqMan assays were used in this study: C____730925, C__34291999, C__27515396, C__31941696, C__31941689, C___1417119, C___1417053, and C__30593278. The detection of loss of heterozygosity in patient DNA samples and hematopoietic progenitor colony DNA was carried out using the Genome-Wide Human SNP 6.0, 50K XbaI, and 10K XbaI Mapping arrays (Affymetrix). Data analysis was performed on the Genotyping Console version 2.1 (Affymetrix) and the dChip software packages (http://biosun1.harvard.edu/complab/dchip).

3.3.3 Analysis of multiple occurrence of JAK2-V617F

Granulocyte genomic DNA of patients heterozygous for rs12343867 and positive for JAK2-V617F was used for the assay (n=109). Allele-specific PCR was performed with primers 5'-GTTTCTTGTTTTAAATTATGGAGTATGTT-3' and 5'-AAAGCATGGGGTACGATTTATAC-3' using AmpliTaq Gold polymerase in Gold buffer with 1.5 mM magnesium chloride (Applied Biosystems). PCR was performed using the 2720 Thermal Cycler (Applied Biosystems) with an initial denaturation step at 95°C for 10 minutes, followed by 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds as well as a 10 minute final extension at 72°C. Next, 10 ng of PCR product were used for TaqMan assays to determine the *JAK2* allele that acquired the JAK2-V617F mutation. This analysis was performed using the commercial TaqMan assay C__31941689 and the 7900HT Thermal Cycler (Applied Biosystems) according to the manufacturer's recommendation.

3.3.4 Statistical analysis

Statistical analysis of SNP association was computed using the PLINK software (http://pngu.mgh.harvard.edu/~purcell/plink) (179). Allelic and genotypic association tests were conducted applying the chi-square test, Fisher's exact test and Cochran-Armitage trend test by comparison of different case-control groups. The corresponding unadjusted *P* values remained significant after correction for multiple testing using the Bonferroni, Holm, Sidak and FDR control methods. Haplotype phases and haplotype associations were determined and the derived linkage disequilibrium (LD) values were visualized using the Haploview software (www.broad.mit.edu/mpg/haploview). Chi-square test for the distribution of *rs12343867* alleles that acquired the JAK2-V617F mutation in heterozygotes was performed using the R statistical package (http://www.r-project.org). Odds ratio calculations for SNP genotype associations were performed using the SNPStats statistical web tool (http://bioinfo.iconcologia.net/SNPstats) (180).

3.4 Familial MPN

3.4.1 Patient characteristics

A total of 982 consecutive patients with apparent sporadic MPN that were diagnosed and followed from 1973 to 2010 at the Department of Hematology, Fondazione IRCCS Policlinico San Matteo of Pavia, were interviewed for familial history of MPN. Among these, DNA for molecular evaluation was available from 772 cases (79%) that were included into the study. Patients were defined as familial cases if two or more individuals within the same pedigree were affected by MPN. The diagnosis was made according to the criteria in use at the time of the first observation, being the PVSG, WHO 2001 or the WHO 2008 diagnostic criteria (147,177,178). Blast phase was defined by bone marrow or peripheral blood blast cell count equal to or higher than 20% (147). Patients with acute myeloid leukemia (AML) were not included into the analysis of malignancies other than MPN as the blast phase is considered a progressive stage of MPN. Solid tumors and lymphoproliferative disorders occurring both, before and after the diagnosis of MPN, were considered as associated malignancies. The diagnosis of solid tumors was based on biopsies of the suspected lesion and the diagnosis of lymphoproliferative disorder was made according to the WHO 2008 criteria. Subjects with normal hemogram (n=43) or a hematological reactive condition (n=160) were used as a demographically matched control population (n=203). Of these, 34 individuals were diagnosed with reactive leukocytosis and 126 with reactive erythrocytosis. Peripheral blood from patients was sampled after written informed consent was obtained. Blood sampling, cell fractionation and DNA isolation from granulocytes and CD3-positive T lymphocytes were conducted as previously described. This study was approved by the institutional ethics committee (Comitato di Bioetica, Fondazione IRCCS Policlinico San Matteo) and the procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000.

3.4.2 *JAK2* mutation analysis

Molecular studies were performed in all patients with familial (n=88) and sporadic (n=684) MPN with DNA available. The *JAK2*-V617F mutation burden was assessed using a quantitative PCR-based allelic discrimination assay, as previously described with the following modifications (181). All reactions were carried out on a RotorGene 6000TM Real-Time Analyzer on a 100-well Gene Disk (Corbett Life Sciences) in a final volume of 12 µl containing 1x Brilliant SYBR Green QPCR master mix (Stratagene) and 300 nM of both, forward and reverse primers. Serial dilutions starting at 80 ng/µl and ending at 0.4 ng/µl of both, a wild type (WT) and a fully mutated DNA sample were used to construct standard curves from which the *JAK2*-WT and V617F quantities were calculated for each sample. Results were expressed as the percentage of V617F alleles among total *JAK2* alleles. *JAK2*-V617F mutation analysis using allele-specific PCR amplification was conducted as described under Methods 3.1.2.

3.4.3 SNP genotyping

Two tagging SNPs in linkage disequilibrium (*rs10974944* and *rs12343867*) were used in order to determine the *JAK2* gene haplotype. Genotyping was performed using commercially available TaqMan assays (C__31941696 and C__31941689, Applied Biosystems) according to standard protocols and data were analyzed on the 7900HT Real-Time PCR instrument (Applied Biosystems). Identification of cytogenetic aberrations in three familial MPN patients of the same pedigree was carried out by high-resolution SNP genotyping using the Genome-Wide Human SNP 6.0 Mapping arrays and data were analyzed with the Genotyping Console software version 3.0.2 (Affymetrix).

3.4.4 Sequence analysis

Sequence analysis of *TET2, CBL* and *MPL* genes was conducted in familial MPN patients with sufficient amounts of DNA available (n=88). Nine coding exons of the *TET2* gene as well as exons 8 and 9 of *CBL* that carry most known *CBL* mutations and exon 10 of the *MPL* gene were sequenced using the BigDye Terminator v3.1 Cycle Sequencing chemistry and the 3130xl Genetic Analyzer (Applied Biosystems) according to standard protocols (182,183). Data analysis was performed using the Sequencher software version 4.9 (Gene Codes). The sequences of primers used for PCR amplification and sequence analysis are shown in Table 9.

3.4.5 Statistical analysis

Statistical analysis of SNP association in familial and sporadic MPN was performed using the R statistical software in conjunction with the SNPassoc package for R version 1.6-0 (184). Individuals with reactive conditions (n=203) were used as a demographically matched control population. Allelic association for *rs10974944* comparing the allele frequencies in familial and sporadic MPN was carried out using the PLINK statistical program (179).

The approximate penetrance in familial MPN was calculated assuming that all affected members have a “causative hypothetical mutation” in heterozygous state (dominant type of inheritance). Based on this assumption, the probability of each healthy member to carry the mutation was calculated. As an example, the healthy child of an affected member has a probability of 0.5 for carrying the MPN-causing mutation, whereas a sibling has a probability of 0.5 as well. This number was summed up to an artificial “number of healthy carriers”. Then, the number of affected members was divided by the sum of the number of affected members and the number of healthy carriers, resulting in the value of penetrance in familial MPN. The penetrance of the *JAK2* GGCC haplotype was estimated by dividing the prevalence of MPN in the general population (10^{-4}) by the frequency of *JAK2* GGCC haplotype carriers, which was approximately 50% when homozygous and heterozygous carriers were taken together.

Malignancies occurring either before or after the diagnosis of MPN were recorded and statistical analysis was carried out using the programs STATA/SE version 9.2 and STATISTICA version 8.0. The relative risk of developing cancer among familial MPN patients compared to sporadic cases was estimated by means of odds ratio (OR). Statistical adjustments by age and *JAK2* haplotype were performed using the Mantel-Haenszel method. Patients were grouped into four age categories with approximately equal numbers of subjects in each group to determine the differences in occurrence of malignancies according to age at MPN diagnosis. The same analysis was conducted after patients were categorized into three groups with similar numbers of study subjects according to age at last follow-up. Comparison of the incidence of malignancies after MPN diagnosis in familial and sporadic MPN patients was estimated using incidence rate ratio (IRR) calculations. Age and *JAK2* haplotype-adjusted Mantel-Haenszel IRR estimates were calculated in order to correct for the influence of age at MPN diagnosis and *JAK2* haplotype on the statistical evaluation. Kaplan-Meier estimation and logrank test were conducted in order to define the difference in cancer-free survival between familial and sporadic MPN.

4 RESULTS

4.1 Deletions of chromosome 13q

4.1.1 The frequency of chromosome 13q deletions in MPN

Deletions of the long arm of chromosome 13 (del13q) have been previously reported in literature applying cytogenetic methods such as chromosome banding and fluorescence in situ hybridization (FISH) (114). In order to determine the frequency of del13q in our single-institutional cohort of 367 MPN patients, we took advantage of polymorphic sites across the previously defined common deleted region of del13q (114). A comparative screening of polymorphisms such as microsatellites and SNPs was performed in granulocyte (tumor tissue) and mononuclear cell or buccal DNA (control tissue) in order to detect loss of heterozygosity (LOH) at the respective position. The complete or partial loss of one allele indicates the presence of a genetic defect such as a deletion or uniparental disomy in all (full LOH) or a fraction (partial LOH) of granulocytic cells (Figure 1A). For this analysis, microsatellite PCR assays covering the entire known CDR of chromosome 13q deletions in myeloid malignancies were designed (Figure 1B).

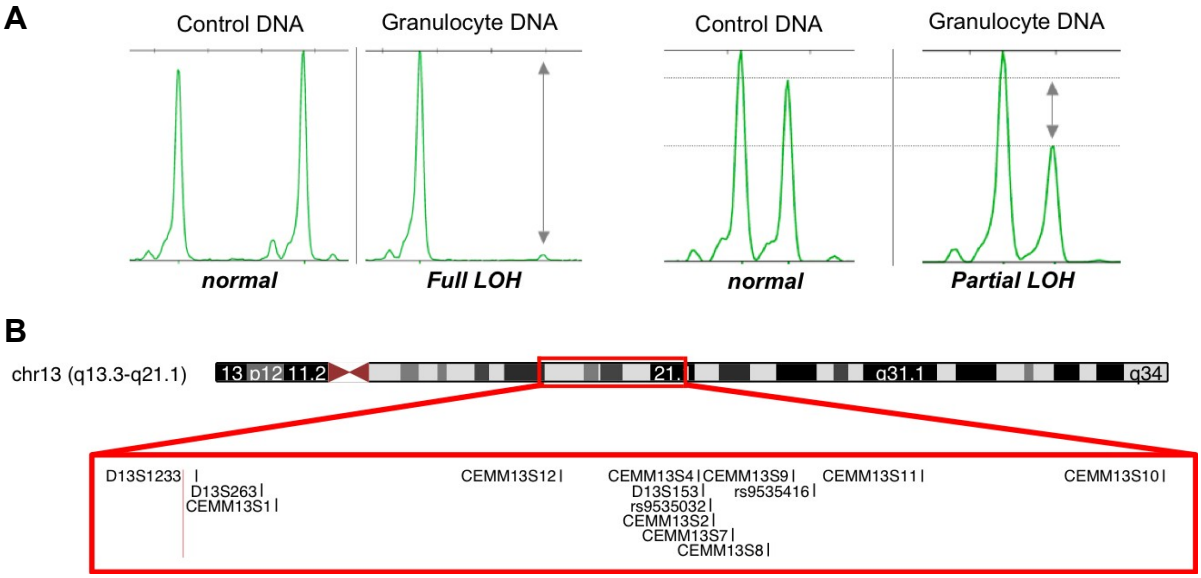


Figure 1. Del13q screening in MPN patients using microsatellite marker PCR. A. Electropherogramm of microsatellite marker PCR. Left panels of both graphs show equal peak heights

for the two alleles of a microsatellite marker PCR in the control sample (buccal or mononuclear cell DNA). Right panels depict granulocytic DNA from the same patient showing a complete loss of one of the microsatellite marker alleles (full LOH) or a partial loss (partial LOH). A partial decrease in peak height is detectable in the granulocyte compared to the control DNA sample, due to the fact that only a fraction of granulocytes carries a deletion at this particular microsatellite marker position. **B.** All microsatellite and SNP markers used for the del13q LOH screening in this study. Known microsatellite markers (e.g. D13S1233) are shown as well as newly designed markers (e.g. CEMM13S1) and SNPs (e.g. *rs9535416*), covering the entire known del13q CDR in MPN.

Importantly, the identification of LOH requires heterozygosity for the investigated marker and thus, these assays are limited to the number of individuals that carry both alleles of the respective polymorphism. In order to increase the informativity of the microsatellite LOH screening, two single nucleotide polymorphisms (SNP) were added to the LOH analysis, one inside the *RB1* gene region (*rs9535032*) and another one downstream of the *microRNA-15a/16-1* cluster region (*rs9535416*) on chromosome 13q (Figure 2A and B). Figure 2C shows the data obtained by microsatellite marker *CEMM13S8* as an example of the microsatellite LOH screening, while Figure 2D depicts the validation method by performing triplicate microsatellite PCR for each suspected LOH case. The combined LOH screening applying twelve microsatellite markers and two SNPs in 367 MPN patients revealed 23 cases with a partial or full loss of heterozygosity at one or more polymorphic sites (Table 10). Of these, three patients (13%) displayed full LOH whereas twenty cases displayed partial LOH (87%) at one or more marker positions. In order to confirm our LOH data and to fine-map the deleted region on chromosome 13q, we performed high-resolution genotyping using Affymetrix Genome-Wide Human SNP 6.0 arrays in 353 patients of our MPN cohort. The presence of del13q was confirmed in all three cases with full (n=3/3) and in five cases with partial LOH (n=5/20, 25%). Three additional cases with del13q were identified. Thus, the frequency of confirmed del13q cases was determined as 3.1% (n=11/353) in our single-institutional MPN patient cohort.

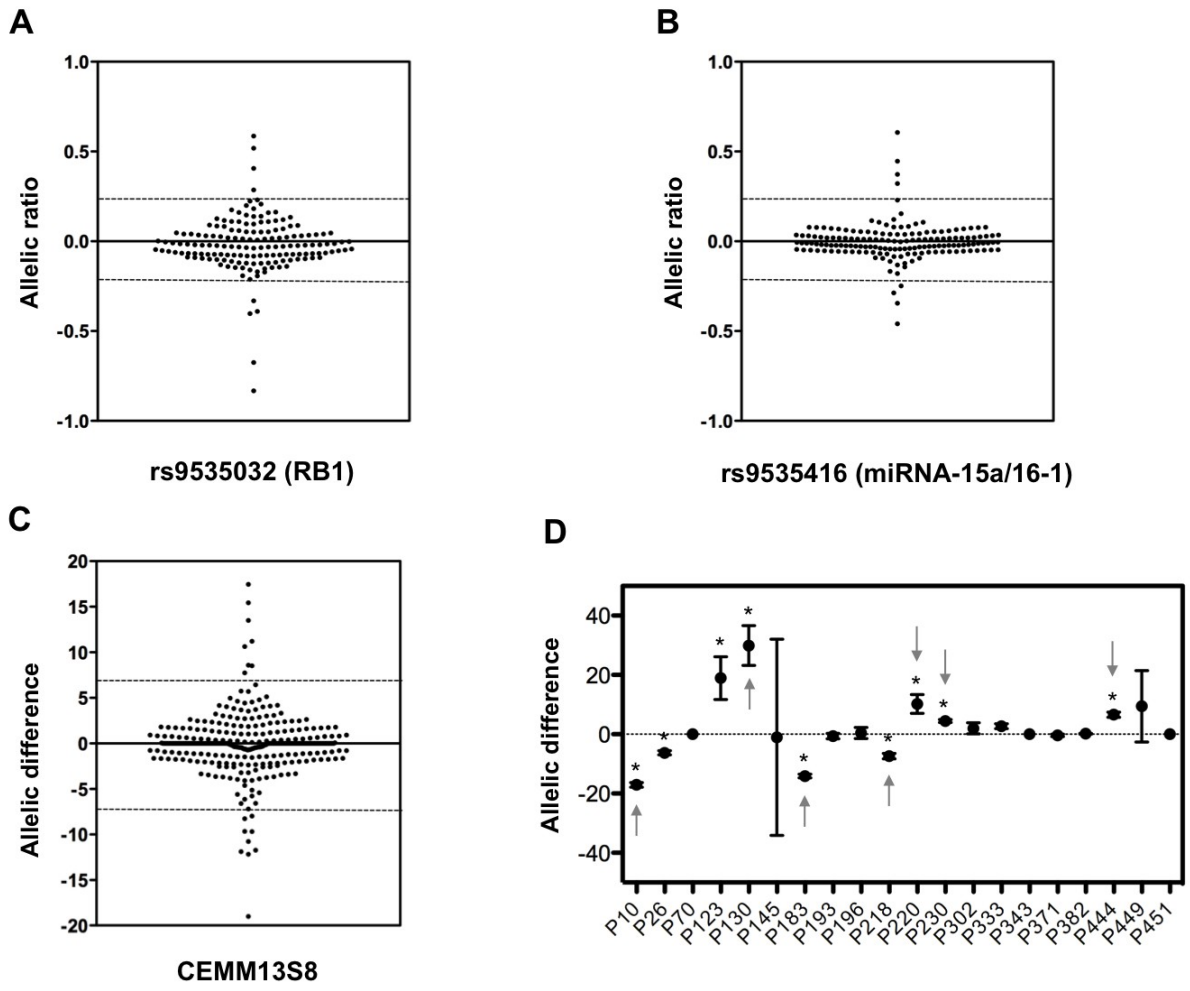


Figure 2. Screening for loss of heterozygosity on chromosome 13q in MPN. A. Loss of heterozygosity (LOH) screening using a SNP in the *RB1* gene locus (*rs9535032*). The ratio between the allelic fluorescence intensities is shown with each dot representing the ratio between the mononuclear cell DNA (control) and the granulocyte DNA (tumor tissue) sample. All dots outside of the dotted line have an altered allelic ratio, indicating a loss of heterozygosity at the respective SNP locus. **B.** Allelic ratio plot of a SNP adjacent to the *microRNA-15a/16-1* gene locus (*rs9535416*). **C.** Example of the results obtained by LOH screening using the microsatellite marker CEMM13S8. Dots represent the difference between the allelic ratio of the control (mononuclear cell DNA) and the tumor tissue (granulocyte DNA). The samples outside of the dotted line show an unequal allelic difference in the two DNA samples. Microsatellite PCR is repeated in triplicates for those suspected LOH cases, as shown in panel D. **D.** Validation of LOH cases by microsatellite PCR. Each reaction is carried out in triplicates, the mean value of allelic differences from three PCR reactions and the respective standard deviations are shown for each of the assessed samples. Cases with a suspected LOH at chromosome 13q are marked with asterisk. Grey arrows indicate cases with a confirmed deletion of chromosome 13q using Affymetrix Genome-Wide Human SNP 6.0 mapping arrays.

In order to further investigate and compare the frequency of del13q among different MPN patient cohorts, we conducted Affymetrix Genome-Wide Human SNP 6.0 array analysis in 59 MPN patients from Vienna, Austria, and 79 MPN patient

samples from Florence, Italy. We identified two additional del13q cases in the Viennese patient cohort as well as three cases from Florence. Thus, the observed frequencies of del13q in MPN were similar to the incidence in our cohort, being 3.4% (n=2/59) in the Viennese cohort and 3.8% (n=3/79) in the MPN patient cohort from Florence. Taken together, deletions of chromosome 13q are recurrent chromosomal aberrations that affect about 3.3% of patients with MPN (n=16/491).

4.1.2 The common deleted region of del13q in MPN

Genomic data of SNP genotyping arrays allows mapping of chromosomal aberrations at high resolution. We performed Affymetrix Genome-Wide Human SNP 6.0 array analysis in order to define and characterize somatic chromosomal changes that contribute to the disease pathogenesis in MPN. The delineation of mapping data from all 16 cases with del13q revealed a deleted chromosomal region of 850 kilo base pairs (kb) that was common to all cases and contained seven genes (Figure 3). This novel common deleted region (CDR) is significantly smaller than the previously reported CDR that was based on data from FISH analysis of 20 patients with various myeloid neoplasms. The study by La Starza et al. defined a CDR for del13q in myeloid neoplasms of about 12.7 mega base pairs spanning the chromosomal region between bands 13q14 to 13q21 and containing about 88 genes (114). Interestingly, the gene that was hypothesized to be the target of del13q in myeloid neoplasms, the well-studied retinoblastoma 1 (*RB1*) tumor suppressor gene, remains contained in the newly defined CDR.

One MPN patient (F430) showed a deletion of chromosome 13q that did not overlap with the described CDR, but delineated a second common deleted region that is shared by 14 of 16 del13q cases identified in this study (Figure 3B). This second CDR spans about 2.5 kb and contains 22 genes including the *MIRN15A*/*16-1* gene cluster. The microRNAs miR-15a and -16-1 are frequently deleted in B-cell chronic lymphocytic leukemia and have been implicated in its disease pathogenesis. However, this second CDR is based on a single case of del13q and thus, the relevance of this chromosomal region in MPN pathogenesis remains unclear.

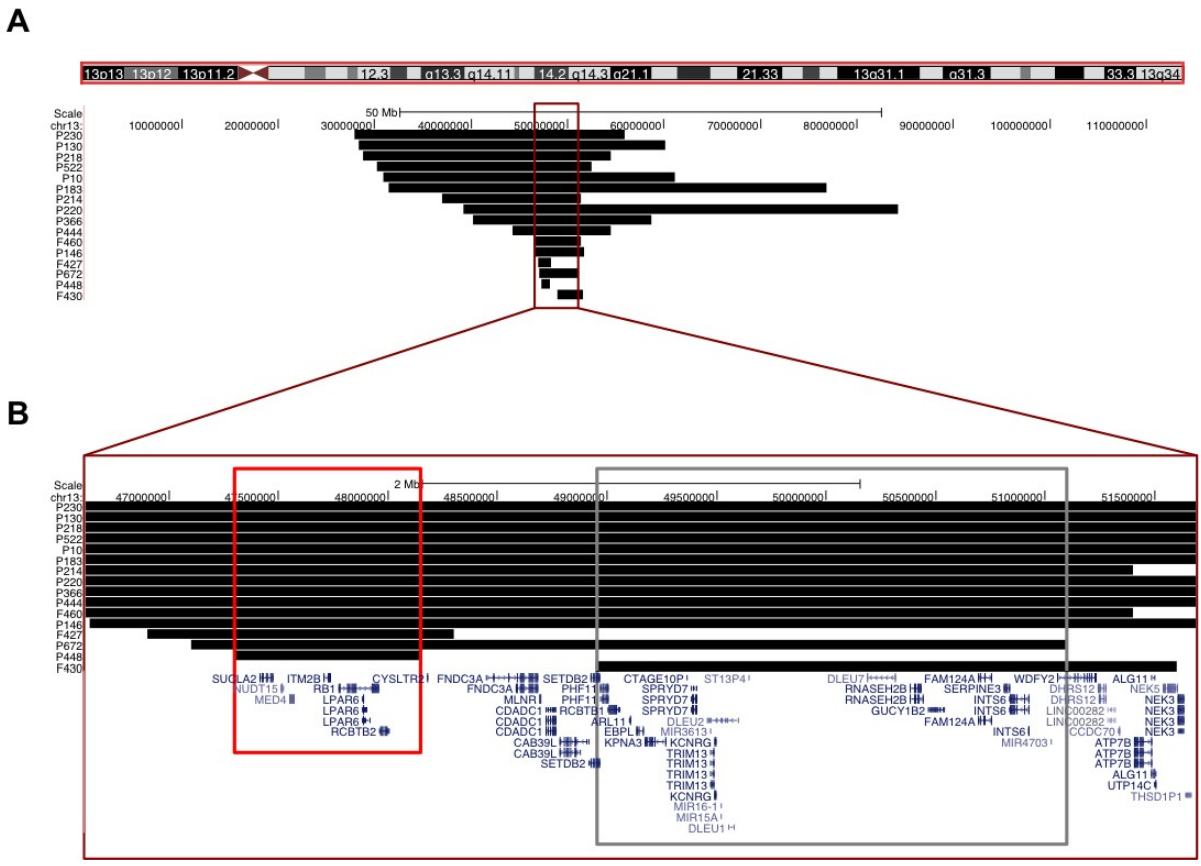


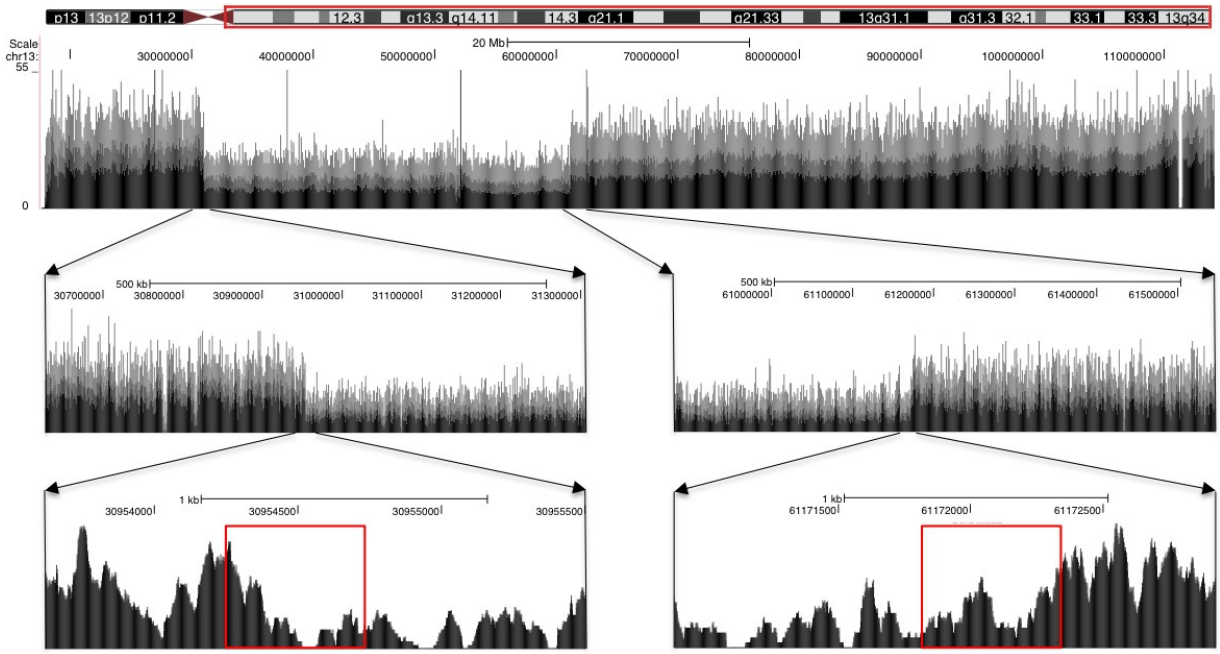
Figure 3. The common deleted region of chromosome 13q deletions in MPN. A. The deleted regions of chromosome 13q in 16 MPN patients based on Genome-Wide SNP 6.0 mapping array data. An overview of chromosome 13 is shown. Black horizontal bars indicate the deleted chromosomal region on 13q for each patient. **B**. Detailed view of the affected chromosomal regions. Red rectangle frames the deleted region on chromosome 13q that is common to 15 of 16 assessed cases and includes the tumor suppressor gene *RB1*. Grey rectangle delineates a second CDR that is shared by 14 of 16 del13q cases and harbors the *microRNA-15a/16-1* genes. This figure was created based on the graphic output of the UCSC Genome Browser (http://genome.ucsc.edu).

4.1.3 The breakpoints of del13q in MPN

In order to further characterize 13q deletions in MPN, we performed whole genome sequencing in a patient with del13q (P10) using the Illumina HiSeq 2000 platform. As the amount of granulocyte genomic DNA from this patient was limited, first whole genome amplification was performed. Amplified granulocyte DNA was next processed through DNA fragmentation, adaptor ligation, cluster generation and synthesis sequencing according to the manufacturer's guidelines. The obtained sequence reads were aligned to the NCBI36/hg18 reference genome and the

coverage was determined as number of reads per base. Figure 4 depicts the sequence coverage of each base of chromosome 13q in sample P10. Interestingly, we observed that the number of reads per base was lower in the deleted region of chromosome 13q as previously determined by SNP microarray mapping. Moreover, we could delineate two regions of each less than 1 kb that were suspected to harbor the breakpoints of the deletion of chromosome 13q in this patient (Figure 4A). To determine the breakpoints of this particular del13q event, we designed three PCR primer pairs covering the suspected breakpoint regions, each with a forward primer upstream of the centromeric breakpoint and a reverse primer downstream of the suspected telomeric breakpoint. The PCR reactions were designed in a way that amplification of the deleted chromosome would result in PCR products of about 500 to 600 bp lengths, whereas amplification of the undeleted allele would not take place due to the long chromosomal distance between the primer pairs of more than 3 Mb in this particular case. Using different combinations of three primer pairs, a total of nine PCR reactions of the del13q breakpoint region were obtained. Following PCR amplification, Sanger sequencing was performed and the results were aligned to the reference sequence using the UCSC Genome Browser. The results of del13q breakpoint sequencing in granulocyte genomic DNA of patient P10 are shown in Figure 4B. Both deletion breakpoints were located in gene-poor regions and did not result in a partial gene loss or a fusion gene (data not shown).

A



B

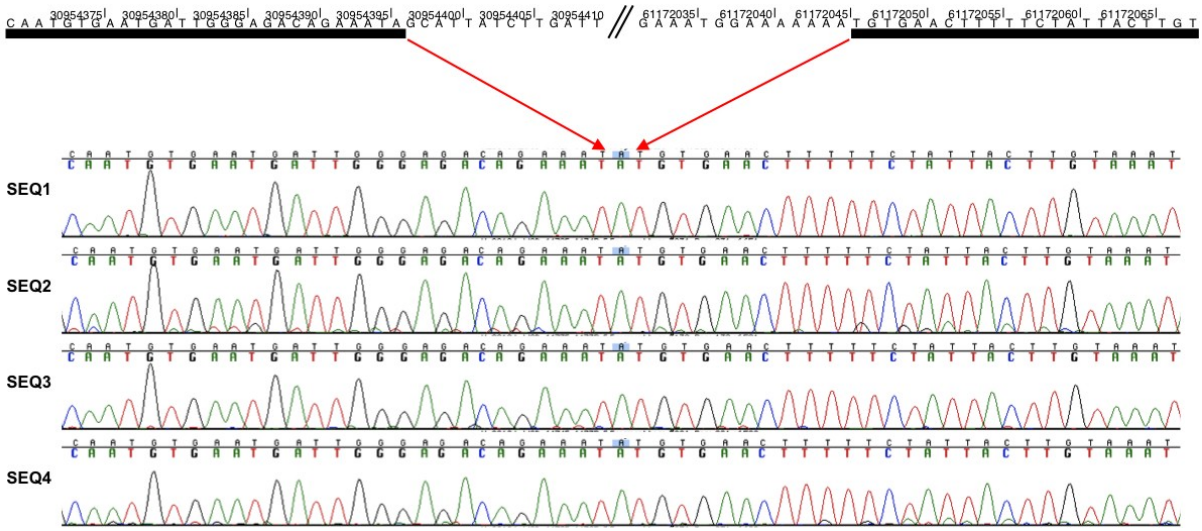


Figure 4. Whole genome sequencing and del13q breakpoint determination in P10. A. Amplified granulocyte genomic DNA was sequenced and aligned to the reference genome NCBI36/hg18. The number of reads per base (sequence coverage) was determined and visualized using the UCSC genome browser. The coverage for each single base is depicted as grey bars (y-axis), the x-axis represents the physical position on chromosome 13q. The sequence coverage is reduced in the deleted region of chromosome 13q in this patient. Lower panels show a detailed view of the suspected breakpoints of del13q in P10. Red rectangles frame the genomic region of the centromeric and telomeric breakpoints of del13q that were used for PCR primer design for breakpoint sequence analysis. **B.** Detailed view of the genomic sequence of both breakpoints. Upper panel shows the aligned sequences of the breakpoint PCR products (black bars) to the reference genome using the UCSC genome browser. Lower panel depicts the electropherogramms of independent sequencing reactions using four different PCR primer pair combinations (SEQ1-4). Red arrows delineate the breakpoint of del13q in patient P10.

4.1.4 Characteristics of MPN patients with del13q

Table 11 serves a summary of the clinical and genotypic characteristics of the 16 MPN patients with del13q identified in this study. Of note, all del13q cases were diagnosed with PV, PMF or secondary myelofibrosis (sMF) whereas no ET patient with an aberration of chromosome 13q was identified (Figure 5A). Moreover, 12 out of 16 patients del13q (75%) were positive for the JAK2-V617F mutation. Of the four del13q patients that were JAK2-V617F negative, one carried the MPL-W515L mutation whereas none had a mutation of *JAK2* exon 12. Although deletions of chromosome 13q seem to occur mostly in PV and PMF patients that are JAK2-V617F mutation positive, no statistically significant difference in the prevalence of JAK2-V617F between MPN patients with or without del13q was detected (Fisher's exact test, *P*=0.7637). Of the twelve JAK2-V617F positive patients, nine carried a uniparental disomy of chromosome 9p. Thus, 9pUPD represents the most frequent chromosomal aberration in del13q positive MPN patients (9/16, 56%), followed by deletions of chromosome 20q (3/16, 19%) and 4p (2/16, 13%). Other chromosomal abnormalities that were detected in del13q patients included uniparental disomies of chromosomes 1p and 14q, deletions of 2p, 7p, 12q and 18p, and gains of chromosome 3q and 9p. Figure 5C shows the frequencies of these genetic defects among all additional chromosomal aberrations that were identified in del13q patients (n=22). In two cases, no chromosomal defect other than del13q was identified, whereas seven patients had one additional aberration with 9pUPD being the most frequent (5/7, 71%). Six del13q patients presented with two additional genetic abnormalities. A PMF patient (P444) with secondary acute myeloid leukemia (sAML) showed a complex karyotype with three chromosomal defects including del7p, an aberration that was demonstrated to associate with increased risk of leukemic transformation (111). The comparison of 9pUPD frequency in MPN patients with and without del13q revealed that 9pUPD was significantly more frequent in del13q positive than negative patients (Chi-square 7.214, *P*=0.0072). Moreover, the overall frequency of chromosomal defects additional to del13q was higher in patients with the deletion of chromosome 13q than in MPN patients without the deletion (Chi-square 41.67, *P*<0.0001). The results of these analyses are illustrated in Figure 5B.

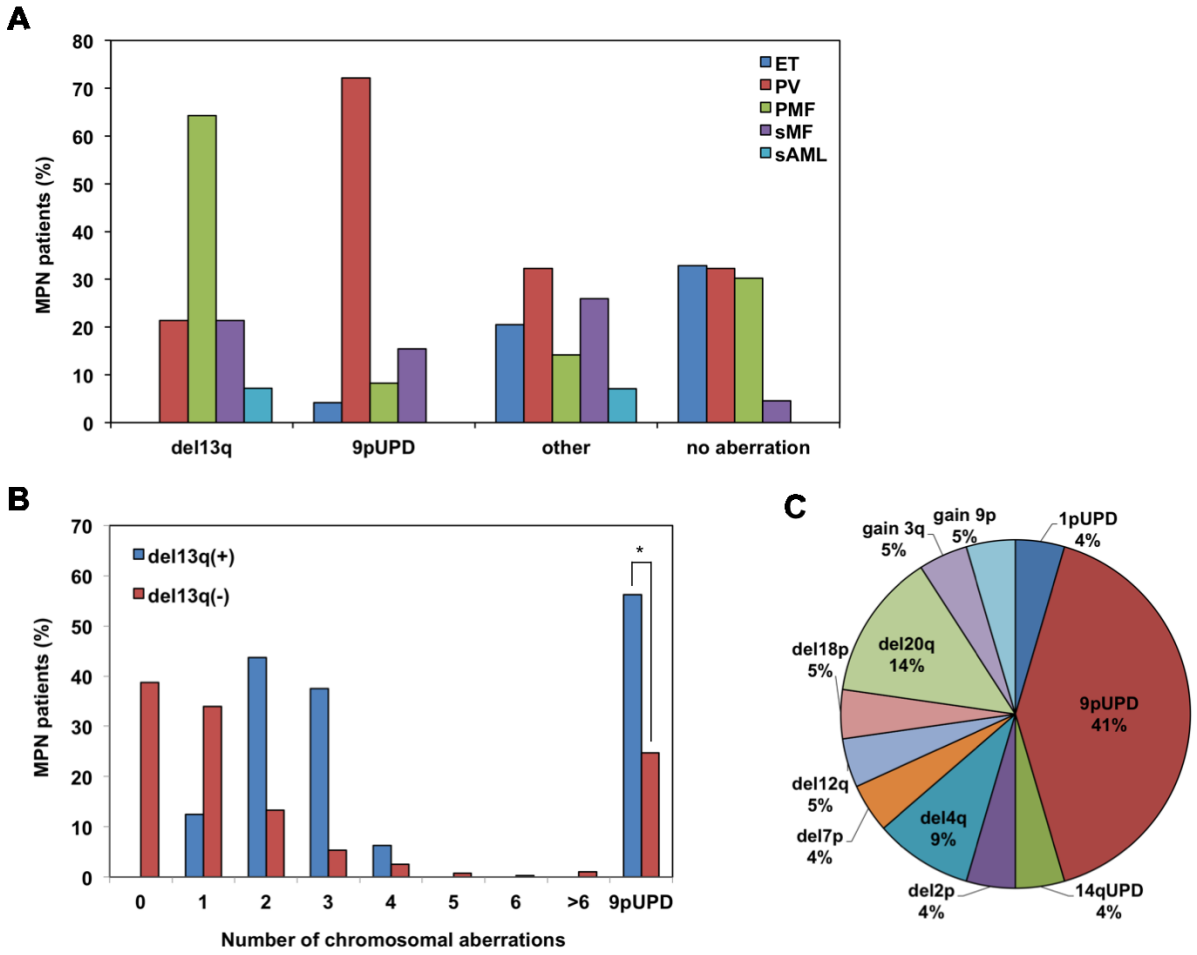
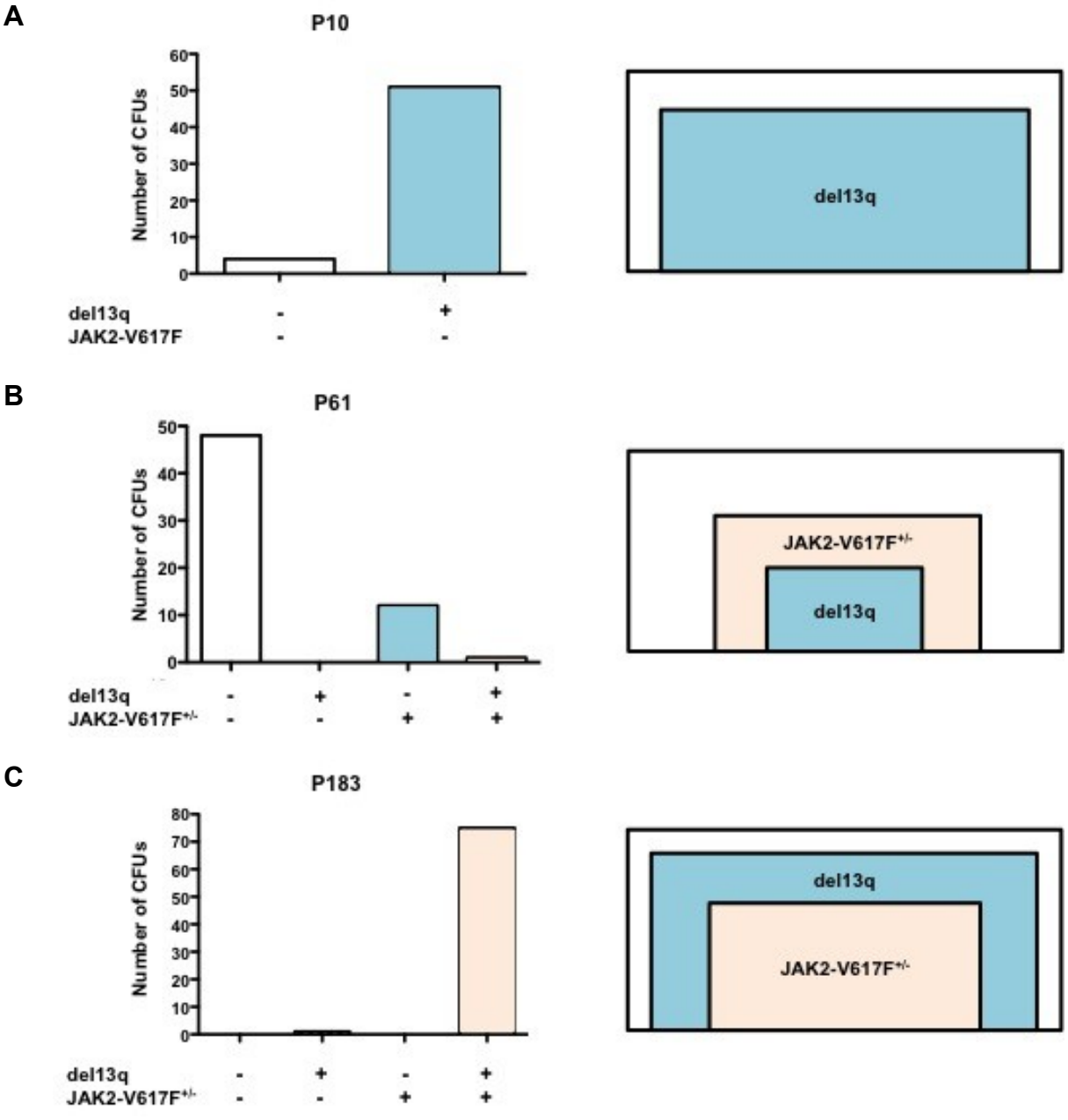


Figure 5. Chromosomal aberrations in MPN patients with and without del13q. A. Frequencies of del13q, 9pUPD, other chromosomal aberrations and the percentage of patients with no chromosomal aberration, grouped according to the diagnostic MPN entities essential thrombocythemia (ET, blue bars), polycythemia vera (PV, red bars), primary myelofibrosis (PMF, green bars), post-ET or post-PV myelofibrosis (secondary myelofibrosis, sMF, violet bars) and secondary acute myeloid leukemia (sAML, turquois bars). The genomic data of 392 MPN patients obtained using Affymetrix Gene-Wide SNP 6.0 mapping arrays is shown. A total number of 16 MPN patients with del13q were identified, 97 patients had 9pUPD, 127 carried chromosomal aberrations other than del13q or 9pUPD and 152 patients presented with a normal karyotype. **B.** The number of identified chromosomal abnormalities as well as the frequency of 9pUPD among patients with (n=16, blue bars) and without del13q (n=376, red bars) is shown. The frequency of additional chromosomal aberrations was higher in patients with del13q than in those without (Chi-square 41.67, *P*<0.0001). Most patients with del13q carried at least one (44%) or two (38%) additional chromosomal defects, with a significantly higher frequency of 9pUPD compared to patients without del13q (**P*=0.0072). **C.** Pie chart indicating the frequencies of chromosomal defects in MPN patients with del13q. A total number of 22 additional genetic aberrations were identified in del13q positive cases, of which 9pUPD was the most frequent (n=9, 41%), followed by del20q (n=3, 14%) and del4q (n=2, 9%).

4.1.5 Clonal hierarchy of chromosome 13q deletions

Although most patients with del13q carry the JAK2-V617F mutation, it remains unclear whether the acquisition of del13q occurs before or after the JAK2-V617F mutation and whether it is acquired in the same or a different hematopoietic cell clone. Moreover, it is known that the JAK2-V617F In order to investigate the relationship between del13q and the JAK2-V617F mutation we performed colony-forming cell assays. Peripheral blood mononuclear cells (PBMNCs) were isolated from del13q positive MPN patients and plated in clonogenic methylcellulose media with or without erythropoietin. Following 14 days of culture, single hematopoietic colonies representing distinct progenitor clones were isolated and DNA was extracted. Genotypic analysis of single hematopoietic colonies was performed in order to decipher the clonal composition of the patient's progenitor cell pool. Figure 6 shows a representative selection of the obtained colony structures from MPN patients with del13q. Some cases showed a simple clonal composition such as the presence of del13q positive colonies without positivity for any of the other assessed genotypic markers (Figure 6A). In other patients, double-positive colonies for del13q and JAK2-V617F existed, with del13q being acquired either before (Figure 6B) or after the JAK2-V617F mutation (Figure 6C). Alternatively, del13q and the JAK2-V617F mutation could be acquired in two different hematopoietic cell clones (Figure 6D). One patient showed a more complex clonal structure indicating that two distinct JAK2-V617F positive clones co-existed: one clone that first acquired del13q and subsequently gained the JAK2-V617F mutation, whereas the other clone was JAK2-V617F positive, but did not carry a deletion on chromosome 13q (Figure 6E). The latter was further shown to harbor a small subclone carrying a deletion on chromosome 20q. This finding served further evidence that this patient carried two independent JAK2-V617F positive hematopoietic cell clones and raised the hypothesis that the JAK2-V617F mutation could be acquired multiple times in the same patient. We identified a second case with a complex clonal structure that showed one clone with the JAK2-V617F mutation on the background of del13q and a second independent JAK2-V617F positive clone as well as colonies that were double-positive for JAK2-V617F and a deletion on chromosome 20q (Figure 6F). Thus, we conclude that there is no sequential or hierarchical order of acquisition for deletions on chromosome 13q. Del13q can either present as the sole chromosomal

defect, can be succeeded by the acquisition of the JAK2-V617F mutation or can occur as a secondary genetic aberration in a JAK2-V617F positive hematopoietic progenitor clone.



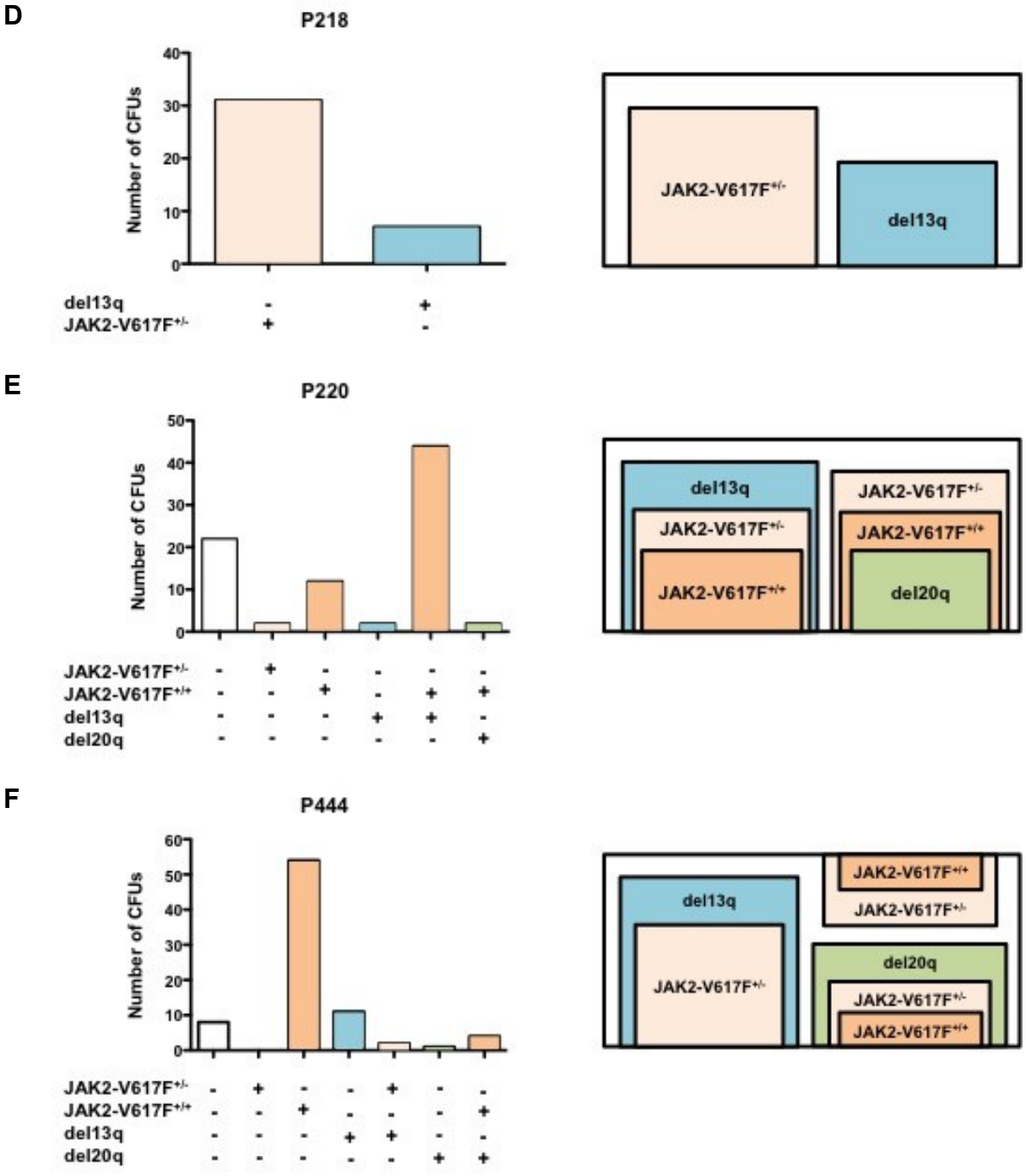


Figure 6. Clonal structures of hematopoietic progenitor cell colonies from MPN patients with del13q. Genotype analysis of single hematopoietic colonies using PCR-based assays for the detection of the JAK2-V617F mutation, del13q and del20q. The left panels depict the number of colonies that are heterozygous (JAK2-V617F$^{+/-}$) or homozygous (JAK2-V617F$^{+/+}$) for the JAK2-V617F mutation and the colonies that carry chromosome 13q (del13q) or 20q deletions (del20q). Right panels illustrate the respective clonal structure of the analyzed hematopoietic progenitor colonies. **A.** The clonal structure of a MPN patient, who carries del13q in most of the analyzed colonies without positivity for any of the other assessed markers, is shown. **B.** Clonal structure with JAK2-V617F mutation positive colonies and a subsequent acquisition of del13q in a V617F positive hematopoietic clone. **C.** Representative case of del13q as the first genetic aberration, followed by the acquisition of JAK2-V617F. Del13q is the sole chromosomal defect in a small number of colonies, whereas the

majority is positive for both, del13q and JAK2-V617F. **D.** MPN patient with two distinct disease clones, one harboring a deletion on chromosome 13q and the other being positive for the JAK2-V617F mutation. **E.** Complex clonal structure with two distinct clones and subsequent acquisition of further genetic defects. The majority of colonies shows positivity for del13q and homozygous JAK2-V617F. A smaller fraction of colonies is negative for del13q and harbors the JAK2-V617F mutation, while some of the V617F-homozygous colonies additionally carry a deletion on chromosome 20q, indicating two independent acquisitions of the JAK2-V617F mutation. **F.** Patient with a complex clonal structure suggesting multiple acquisitions of the JAK2-V617F mutation. Most of the hematopoietic progenitor colonies are homozygous for the JAK2-V617F mutation. Additionally, colonies with sole del13q and del20q are detectable as well as colonies that carry both, del13q or del20q and the JAK2-V617F mutation.

4.1.6 Analysis of the putative target genes of chromosome 13q deletions

Two types of genes were identified to play a causative role in cancer biology: proto-oncogenes and tumor suppressor genes. Oncogenes promote the development of cancer when activated, whereas tumor suppressor genes prevent cancer formation under normal conditions and need to be inactivated for tumor formation. Accordingly, genetic alterations such as mutations or deletions that cause inactivation of tumor suppressor genes lead to abnormal cellular function and tumorigenesis. It was initially thought that tumor suppressor genes would exclusively act in a recessive manner and that complete tumor suppressor gene inactivation is necessary to cause a phenotype. However, investigations in patients with retinoblastoma, a dominant cancer susceptibility syndrome with cancerous degeneration of retina cells, revealed a mutation in the retinoblastoma 1 (*RB1*) gene in hereditary cases of this syndrome. Interestingly, the inherited *RB1* mutation was associated with a high probability to acquire a second genetic defect that would inactivate the remaining allele of *RB1* and thus, initiate tumor formation. This “two-hit hypothesis” of tumorigenesis was postulated by Alfred Knudson and served as a paradigm of tumor suppressor gene function in cancer development (185). The results of further investigations suggested that there are two classes of tumor suppressor genes, namely gatekeepers and caretakers (186). Gatekeeper tumor suppressors control cell proliferation via negative regulation of pro-apoptotic signaling pathways (e.g. *PTEN*) or cell cycle inhibition (e.g. *RB1*). On the other hand, caretakers such as the well-known tumor suppressor gene *p53* prevent tumorigenesis by maintaining genome stability. Investigations in patients with Li-

Fraumeni syndrome led to the identification of various causative germline mutations in the *p53* tumor suppressor gene, but only about 60% of cases showed complete tumor suppressor inactivation with genetic defects in both of the *p53* alleles (187). Further functional analysis in mouse models revealed that heterozygous *p53* knockout animals also developed tumors as did the homozygous knockout mice, but with either heterozygous or homozygous aberrations of *p53* (188). Interestingly, tumors with only one aberrant *p53* allele occurred at later time points than those with homozygous *p53* mutations. Taken together, these results indicated that functional loss of one *p53* allele is sufficient to initiate cancerogenesis, but results in a milder phenotype than in case of a complete tumor suppressor inactivation – a phenomenon referred to as “haploinsufficiency”. In haploinsufficient genes, one functional copy is not enough to maintain protein production at a level that is sufficient for normal cell function (189). Thus, monoallelic alterations of haploinsufficient tumor suppressor genes such as deletions of the respective chromosomal region, loss-of-function mutations or inactivation via epigenetic silencing can promote malignant degeneration.

In this study, we identified deletions of chromosome 13q as recurrent genetic aberrations in patients with MPN and defined a common deleted region that contains 7 genes including the tumor suppressor gene *RB1*. In order to further elucidate the genetic mechanisms that underlie the pathogenesis of MPN we performed functional analysis of all 7 del13q CDR genes. As *RB1* was suggested a putative target gene of del13q, we specifically aimed to define the pathogenetic role of this tumor suppressor in MPN. Interestingly, all deletions of chromosome 13q detected in MPN patients were hemizygous alterations, whereas no case with a biallelic 13q deletion was identified. However, inactivating genetic alterations in the remaining target gene allele such as microdeletions, mutations or epigenetic mechanisms that cannot be detected using SNP mapping arrays might be present. Thus, further genomic and functional analyses were performed taking both pathogenetic mechanisms into account: complete inactivation and haploinsufficiency of the putative target genes of del13q (Figure 7).

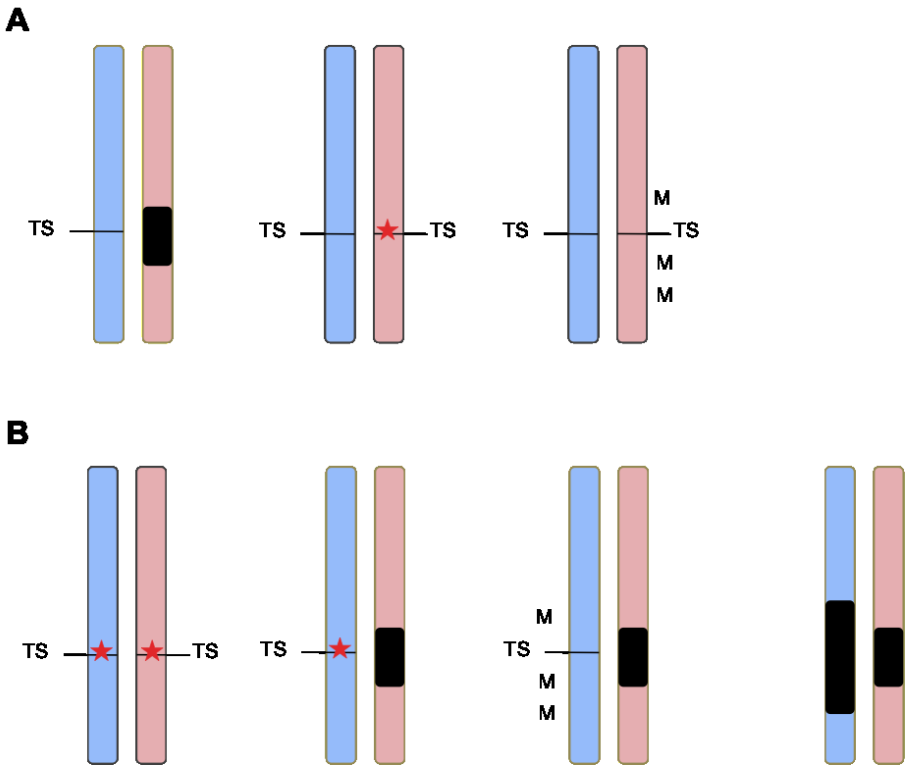


Figure 7. Mechanisms of tumor suppressor gene inactivation. A. Inactivation of a single tumor suppressor gene allele (via deletion, mutation or epigenetic silencing) can result in a neoplastic phenotype due to haploinsufficiency. **B**. Two genetic defects affecting both gene copies are necessary to establish complete tumor suppressor inactivation and to initiate a disease phenotype. The blue and pink bars depict the paternal and maternal chromosomes, the tumor suppressor gene locus (TS) is shown with a black line and black bars indicate deleted chromosomal regions. Mutations (red stars) and altered methylation (M) are depicted as further inactivating mechanisms.

4.1.7 Gene expression analysis of the del13q CDR genes

The results of high-resolution mapping of chromosome 13q deletions in MPN using Genome-Wide SNP 6.0 arrays delineated a del13q common deleted region of about 850 kb containing a total number of 7 genes. In order to elucidate whether the del13q CDR genes are expressed in hematopoietic cells, we performed gene expression analysis of 6 del13q CDR genes in murine primary bone marrow cells. Expression analysis in RNA samples of murine lineage depleted bone marrow cells, lineage positive bone marrow and cells of the murine hematopoietic cell line BaF3 was performed using real-time PCR. The obtained results showed that all examined genes of the newly defined del13q CDR are expressed in murine hematopoietic cells (Figure 8).

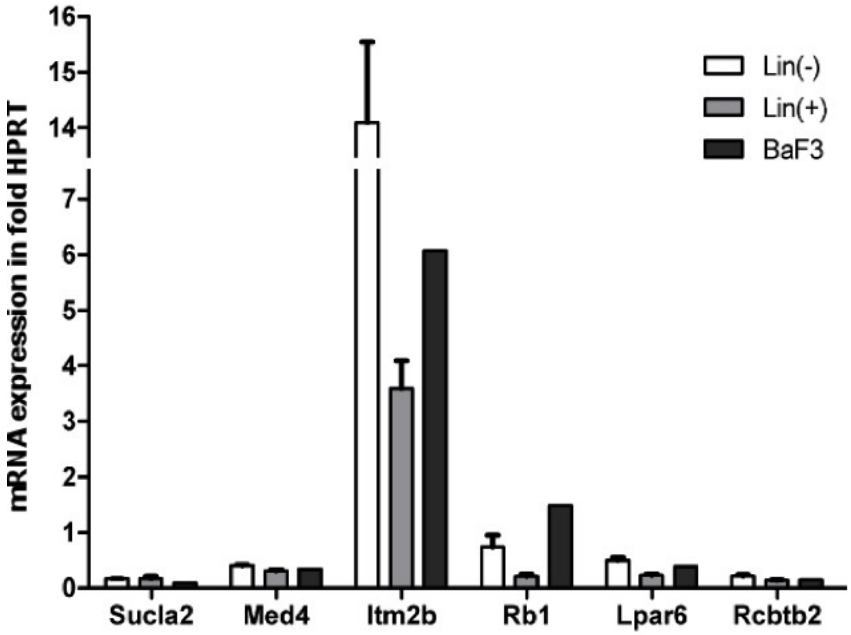


Figure 8. Gene expression analysis of the del13q CDR genes. Expression of mRNA is shown in fold of the housekeeping gene *HPRT*. White bars show gene expression of lineage depleted murine bone marrow cells (Lin(-)), grey bars indicate lineage positive bone marrow cells (Lin(+)) while black bars show respective gene expression in cells of the murine hematopoietic cell line BaF3.

The *RB1* gene has been hypothesized as the target of 13q deletions in myeloid neoplasms since the definition of a common deleted region that harbors this tumor suppressor gene. To examine whether the loss of one allele alters the expression of *RB1* we performed gene expression analysis on granulocytic RNA samples of MPN patients with a deletion on chromosome 13q compared to MPN patients without a deletion. Furthermore, RNA samples of CD34-sorted human cord blood cells and granulocytes were included into the analysis in order to obtain data on the gene expression levels of *RB1* at different stages of hematopoietic differentiation. CD34-positive hematopoietic cells are considered as early stem or progenitor cells, whereas the loss of CD34 expression indicates lineage commitment and differentiation. The results of this analysis showed that *RB1* gene expression does not significantly change through hematopoietic cell maturation and, moreover, is not reduced in patients with del13q compared to controls (Figure 9). Thus, *RB1* gene expression is not significantly altered by monoallelic loss due to hemizygous deletions of chromosome 13q.

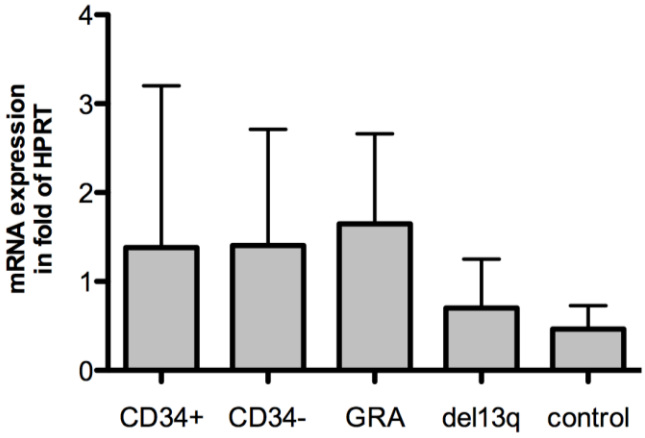


Figure 9. Gene expression analysis of *RB1* in human hematopoietic cells. *RB1* expression of each 5 RNA samples from human cord blood (CD34+, CD34- hematopoietic cells and granulocytes (GRA)) and RNA from granulocytes of 4 MPN patients with a deletion on chromosome 13q (del13q) and 4 patients without a deletion (control) was measured. Expression levels of *RB1* mRNA are shown in fold of expression of the housekeeping gene *HPRT*. Gene expression analysis of *RB1* shows high sample variation and no difference in gene expression throughout hematopoietic cell differentiation or between del13q positive and negative MPN patients.

4.1.8 Sequence analysis of the del13q CDR genes

Point mutations in the remaining allele of genes that are partially lost due to a hemizygous deletion can cause a complete inactivation and loss of function of the respective gene. Thus, sequence analysis was performed in MPN patients with del13q in order to identify potentially inactivating mutations or microdeletions of the del13q CDR genes. First, Sanger sequencing of all 27 exons of the *RB1* gene was conducted on granulocyte genomic DNA of 4 MPN patients with a deletion on chromosome 13q. In a subsequent experiment, sequence analysis of all 7 del13q CDR genes using the Illumina HiSeq 2000 system was performed. An equimolar pool of granulocyte genomic DNA from 8 del13q patients was prepared and processed into a fragmented and ligated DNA library. Cluster generation and synthesis sequencing were conducted on the Illumina HiSeq 2000 sequencing system and the obtained sequence data were aligned to the human reference genome NCBI36/hg18 using the CASAVA software. Equally, the results of this high-throughput sequencing analysis revealed no intragenic missense or splice-site mutations in the 7 genes of the defined del13q CDR, thus indicating that the remaining alleles of these genes are not inactivated by point mutations in patients with del13q.

4.1.9 Epigenetic inactivation of the *RB1* gene

Recent reports in literature show that *RB1* is among the imprinted human genes (190). Genomic imprinting resembles an epigenetic mechanism by which parent-of-origin-specific DNA methylation and gene expression are regulated. Methylation of CpG islands and histone modifications enable the monoallelic expression of genes without sequence alterations, a process important in normal development. As it was recently shown, imprinting of *RB1* is enabled by the evolutionary insertion of a DNA sequence, a processed 5'-truncated pseudogene (*KIAA0649*), which acquired a differentially methylated CpG island (CpG 85) and serves as an alternative *RB1* promotor. Differential methylation at this locus promotes increased gene expression from the maternal *RB1* allele. Kanber et al. performed bisulfate treatment, subcloning and sequencing analysis of whole blood DNA from normal individuals and retinoblastoma patients with *RB1* deletions of known parental origin (190). Their results showed that the CpG island 85 is not methylated in patients with a deletion of the maternal *RB1* gene whereas it is fully methylated in cases with a deletion of the paternal allele. This observation raised the hypothesis that deletions of chromosome 13q might preferentially affect a specific parental allele and that the monoallelic loss of a *RB1* results in a skewed *RB1* expression due to the alteration of epigenetic regulation. Thus, methylation-specific PCR was performed in 11 MPN patients with a deletion of chromosome 13q in collaboration with Deniz Kanber and Bernhard Horsthemke from the Institute of Human Genetics at the University of Essen in Essen, Germany. Methylation-specific PCR on granulocyte and whole blood genomic DNA was conducted in order to quantify methylation of the CpG island 85 in exon 2 of the *RB1* gene. The results of this experiment revealed variable methylation patterns throughout the samples with no obvious preference for a certain paternal allele (Figure 10). Interestingly, a skewed ratio between methylated maternal and unmethylated paternal *RB1* alleles was detected in most samples, which approximately corresponded to the estimated size of the malignant del13q clone in all but one case. In sample P448, methylation of all *RB1* alleles was detected. However, the del13q clone size in this sample was estimated to account for about 30%. This might be due to the presence of full clonality of hematopoietic cells with a yet unknown genetic defect that preceded the acquisition of del13q. In summary, we conclude that 13q deletions do not target a specific parental allele of the *RB1* gene

and that methylation is partially skewed due to the clonal outgrowth of myeloid cells with a deletion on chromosome 13q and a respectively altered methylation status. However, there is no obvious preference towards increased or decreased methylation of *RB1* in MPN patients with del13q, indicating that del13q does not cause abnormalities in methylation-dependent epigenetic mechanisms related to the *RB1* gene.

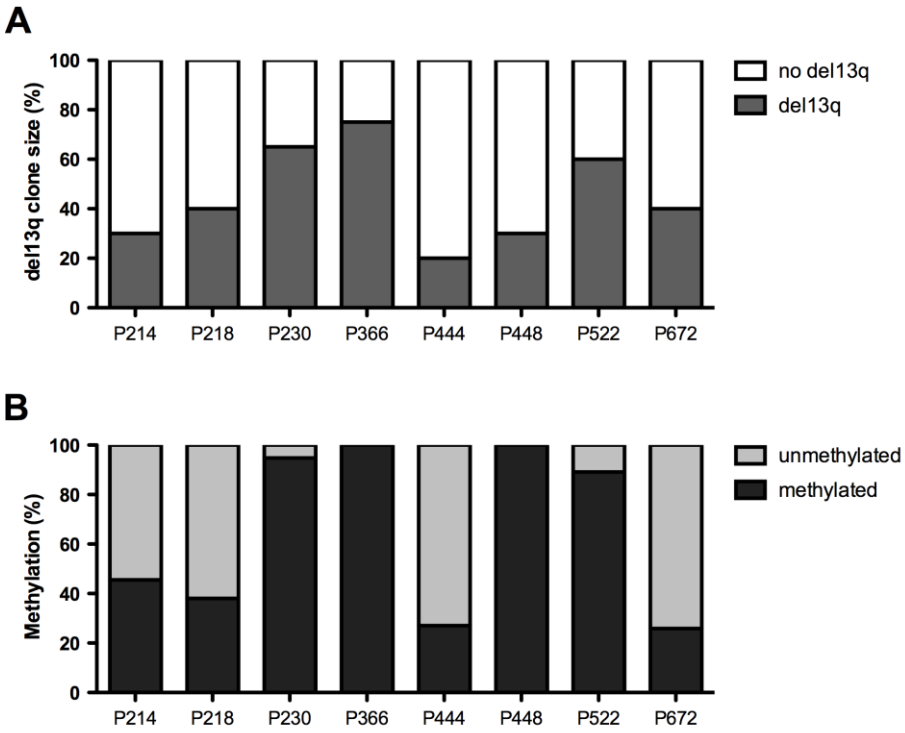


Figure 10. Quantitative methylation analysis of MPN patients with del13q. Estimated del13q clone size and respective methylation analysis of CpG 85 of the *RB1* gene by methylation-specific PCR of 8 MPN patients with del13q. **A.** Estimated clone size of del13q according to the copy number loss detected by Genome-Wide SNP 6.0 mapping arrays. White bars indicate the approximate hematopoietic cell fraction that does not carry a deletion on chromosome 13q, grey bars resemble the estimated size of the del13q-positive disease clone. **B.** Quantitative results of methylation-specific PCR of CpG 85. The percentage of methylation-specific PCR products of unmethylated (light grey bars) and methylated *RB1* alleles (dark grey bars) is shown. The estimated del13q clone sizes and percentages of skewed *RB1* methylation roughly correspond with the exception of sample P448, which shows methylation of all detected *RB1* alleles, although the approximate del13q clone size is only about 30%.

4.1.10 Functional analysis of RB1 deficiency using RNA interference

The *RB1* tumor suppressor gene has been proposed as a putative target of chromosome 13q deletions in MPN. However, no data on the functional impact and cellular outcome of *RB1* loss in hematopoietic cells is available up to date. RNA interference serves as an efficient tool to mimic partial or complete loss of gene expression in cell lines and primary cells. In order to evaluate the hypothesis that monoallelic loss of *RB1* alters hematopoietic cell proliferation and function, we designed specific shRNA transcripts that silence *RB1* gene expression. Human and murine shRNA constructs against *RB1* and control shRNAs (scrambled controls) were integrated into the lentiviral vector pLKO.2. Cells of the human megakaryocytic cell line UT-7/TPO, the murine pro-B-cell line BaF3/EPO and lineage-depleted murine primary bone marrow cells were transfected with each of the corresponding mouse or human *RB1* and control shRNA vectors. Knockdown efficiency was assessed with standard Western blot analysis in case of UT-7/TPO cells (data not shown) and with *Rb1* gene expression analysis using TaqMan Gene Expression assays in BaF3/EPO and murine primary bone marrow cells. XTT proliferation assays were performed in order to examine differences in cytokine sensitivity and cell proliferation of the transfected cell lines and primary bone marrow. After 4 to 5 days of culture at different cytokine concentrations, the amount of viable cells was measured and compared among *RB1* and control shRNA transfected cells. In the thrombopoietin-dependent cell line UT-7/TPO, no significant difference in cytokine sensitivity and proliferation was observed between *RB1*-deficient and control transfected cells (Figure 11A). Although a slight difference in cell proliferation at high TPO concentrations was observed, this is most likely due to variable cell numbers at the time of initial plating and not caused by a difference in cytokine sensitivity. Similar results have been obtained in the murine hematopoietic cell line BaF3/EPO that stably expresses the erythropoietin receptor. Cells transfected with the *Rb1* shRNA construct and those transfected with the control shRNA show overlapping proliferation curves, indicating that there is no difference in cell growth and sensitivity to EPO upon *Rb1* inactivation compared to the control (Figure 11B). In a similar experimental setup, murine lineage-depleted bone marrow cells were transfected with *Rb1* or control shRNA. Proliferation was assessed at increasing concentrations of a cytokine mix including IL-3, IL-6, SCF, Flt3-L and GM-CSF. The results of this

experiment showed a trend towards increased cytokine-dependent proliferation of Rb1-deficient cells at higher cytokine concentrations (Figure 11C). However, the standard deviation of the viable cell count at the respective cytokine levels is high, especially in the control samples, indicating that the number of cells per well was highly variable and that these results are of limited validity. In conclusion, no clear alteration of cytokine sensitivity or proliferation rate between Rb1-deficient and control cells was observed in hematopoietic cell lines or murine primary bone marrow cells. Accordingly, these findings serve evidence that decreased *RB1* expression does not influence sensitivity to growth stimuli or proliferation of myeloid cells.

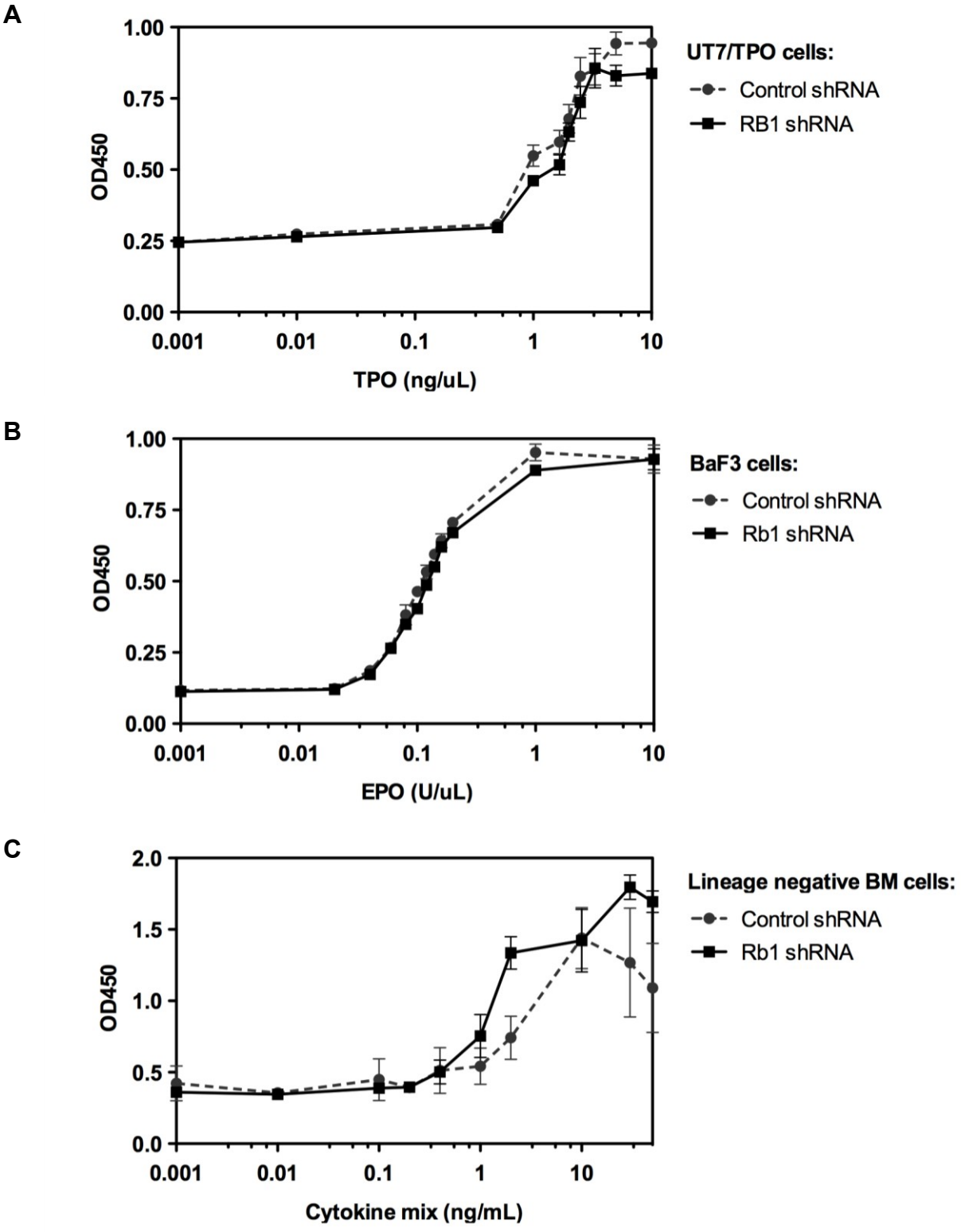


Figure 11. Effect of functional *RB1* inactivation on cytokine-dependent proliferation of hematopoietic cells. Proliferation curves of cell lines and murine lineage-depleted bone marrow cells after inactivation of *RB1* via shRNA knockdown. The number of cells per well is reflected by the spectrophotometric absorbance at a wavelength of 450 nm (OD450). Filled grey circles represent the viable cell count at the respective cytokine concentration in cells transfected with a control shRNA,

filled black squares show the respective values in cells transfected with a human or mouse shRNA targeting the human or mouse RB1 transcript with error bars indicating the standard deviation on quadruplicate samples. **A.** XTT proliferation assay using the human megakaryocytic cell line UT-7/TPO that is entirely dependent on TPO supplementation. RB1-deficiency does not result in a significant difference in TPO-dependent proliferation at different cytokine concentrations compared to the control. **B.** Same experimental setup using the murine pro-B-cell line BaF3/EPO that grows in response to EPO. No altered EPO-dependent cell proliferation was observed upon Rb1 inactivation via a murine shRNA construct compared to the cells transfected with the control shRNA. **C.** Proliferation assay on murine lineage-depleted bone marrow cells using the same shRNA constructs as in B. Rb1-deficient lineage-depleted bone marrow cells show a slight trend towards increased cytokine-sensitivity in comparison to the control, but not consistently at all cytokine concentrations.

4.2 The *JAK2* GGCC haplotype

Performing genotypic analysis of individual hematopoietic progenitor clones, we identified MPN patients with complex colony structures (Figure 6). In one case (P220, Figure 6E) we gained evidence that the JAK2-V617F mutation must have occurred in two independent hematopoietic clones. Single hematopoietic progenitor clones were genotyped for the presence of JAK2-V617F, del13q, and del20q as described above. Using these aberrations as markers of clonality, a total number of six genotypic classes of progenitor clones were identified in this particular patient (Figure 12A, A-F). The majority of progenitor clones was homozygous for JAK2-V617F and positive for del13q (E). A number of colonies carried del13q as a sole chromosomal defect (D), whereas others were exclusively positive for JAK2-V617F in a heterozygous state (B). Furthermore, colonies with homozygous JAK2-V617F without del13q were detected (C) as well as a small number of colonies that was positive for both, homozygous JAK2-V617F and del20q (F). This particular constellation of progenitor colony genotypes led to the hypothesis that the patient must have acquired two independent JAK2-V617F mutations in two different disease clones, one on a del13q positive and the other on a del13q negative clonal background. To confirm these results, we performed cytogenetic analysis using Affymetrix 10K and 50K XbaI microarrays on whole genome amplified DNA of representative hematopoietic progenitor colonies of each genotypic class (Figure 12B).

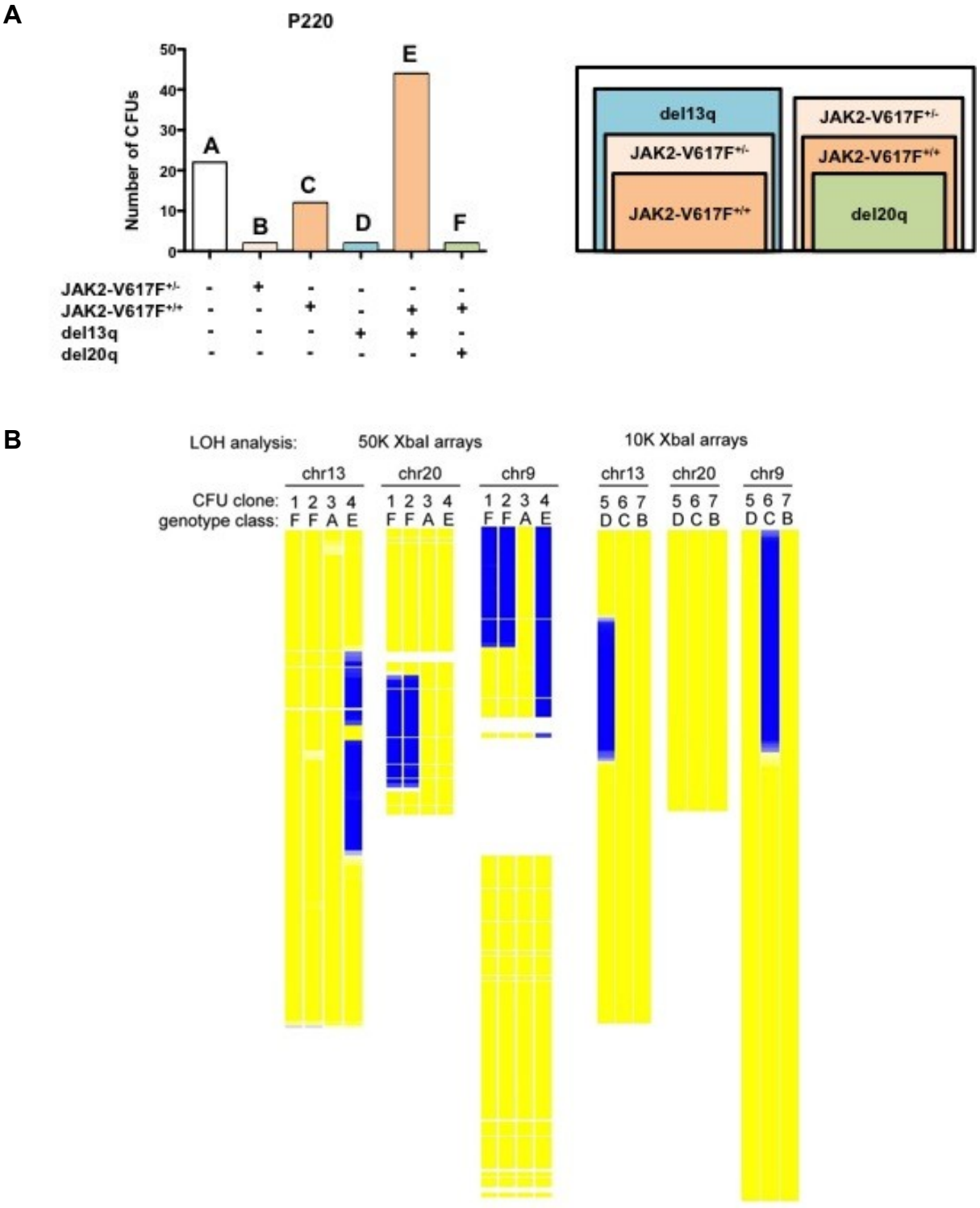


Figure 12. Cytogenetic analysis of hematopoietic progenitor clones in a patient with primary myelofibrosis. A. Genotyping of colony forming units (CFU; primarily consisting of erythroid burst forming units and granulocytic-monocytic CFUs) for the presence of JAK2-V617F (het, heterozygous), uniparental disomy 9p (9pUPD), and deletions on chromosomes 13q (del13q) and 20q (del20q). Colonies with 9pUPD were homozygous for JAK2-V617F. Six genotypic classes were identified (A-F). **B.** Clonal hierarchy of progenitors of the patient (white box) predicted from the identified genotypic classes. Each colored box represents a mutational event. The JAK2-V617F

mutation was acquired in two independent events (V617F): one occurring on the del13q background, the other on wild type (wt) background. **C.** Microarray validation of the six different genotypic classes in seven different CFU clones (1-7). The Affymetrix 50K XbaI and 10K XbaI Mapping arrays were used for probing whole-genome amplified genomic DNA isolated from individually picked CFUs (LOH plots are not in scale between the two array types). Detection of loss of heterozygosity (LOH) was performed using the dChip software. Only chromosomes 13, 20, and 9 (chr13, chr20, chr9) with LOH are shown. Blue color indicates the presence and yellow color the absence of LOH. The del13q deletion breakpoints were identical in clones 4 and 5 (genomic position 39.3-84.2 Mb). The mitotic recombination breakpoints on chromosome 9 were at different positions in clones 1,2 (genotypic class F) and 4,6 (genotypic classes C,E), respectively.

Next, we aimed to elucidate whether multiple acquisitions of JAK2-V617F were recurrent events among MPN patients. A PCR-based assay using a SNP in intron 14 of *JAK2* (*rs12343867*), about 418 bp upstream of the JAK2-V617F mutation locus, was designed in order to determine the frequency of multiple JAK2-V617F acquisitions (Figure 13A and 13B). In patients heterozygous for the SNP, the two alleles could be used to define the chromosome on which JAK2-V617F had occurred. First, an allele-specific PCR assay using a JAK2-V617F specific primer and a primer distal to *rs12343867* was designed. PCR amplification generated products that were obtained only from the *JAK2* allele that carried the V617F mutation and moreover, harbored the *rs12343867* polymorphism. Subsequent genotyping of the obtained PCR products using a TaqMan SNP genotyping assay revealed which of the *rs12343867* (or *JAK2*) alleles had acquired the mutation. In case that the JAK2-V617F mutation was independently acquired on both chromosomes, the allele-specific PCR reaction would contain products of both SNP alleles and their ratio would reflect the population size of the clones (Figure 13B). Genotypic analysis of 213 JAK2-V617F positive MPN patients revealed 109 cases that were heterozygous for *rs12343867* and informative for the assay. Furthermore, we identified three patients (2.8%) who showed positivity for both *rs12343867* alleles and thus, had acquired JAK2-V617F on both alleles of the *JAK2* gene (Figure 13C). Subsequently, the same PCR products obtained from the JAK2-V617F specific PCR reaction were used for subcloning and sequence analysis in order to confirm that the JAK2-V617F mutation had been acquired on both alleles (Figure 13D). Using these methods, we confirmed that the JAK2-V617F mutation can occur at least two times in independent hematopoietic progenitor clones and that about 3% of MPN patients harbor at least two distinct JAK2-V617F positive disease clones. However, these assays are limited

as only patients that are heterozygous for the assayed SNP are informative and cases with independent acquisitions of V617F mutation on the same *JAK2* allele cannot be detected. Thus, the actual frequency of multiple JAK2-V617F mutations might be even higher than the observed frequency using this PCR-based assay.

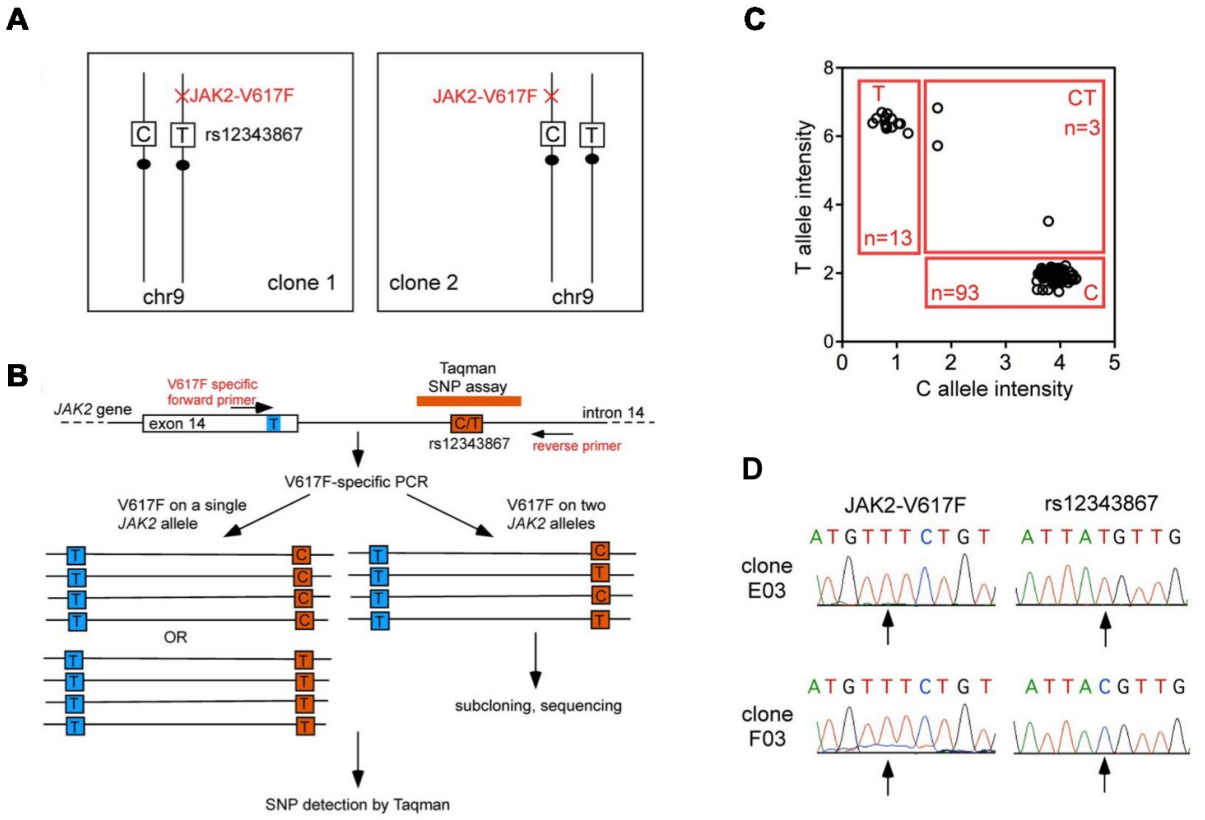


Figure 13. Detection of multiple acquisitions of the JAK2-V617F mutation in exon 14 of the *JAK2* gene in MPN. A. Heterozygosity (C,T) for a SNP (*rs12343867*) in intron 14 allows the discrimination of two independently acquired JAK2-V617F mutations on chromosome 9 (chr9) in two different clones. **B.** Schematic outline of the assay. JAK2-V617F-specific PCR using a JAK2-V617F specific primer (T in blue box) and a primer distal to *rs12343867* (orange box) generates PCR products (horizontal lines) that can be genotyped with a Taqman assay for *rs12343867* (orange horizontal bar). If the JAK2-V617F mutation was acquired on both chromosomes in two independent events, the allele-specific PCR product will be a mixture of the two alleles and their ratio will depend on the population size of the clones. **C.** Detection of the *JAK2* allele that acquired JAK2-V617F in 109 patients (open circles) by Taqman genotyping. Relative fluorescence of probes specific for the C or T genotypes are shown on the x- and y-axis. The number (n) of individual genotype calls (TT, CT, or CC) is shown in red boxes. Patients with CT genotype calls acquired JAK2-V617F independently on two *JAK2* alleles. **D.** Sequence analysis of subcloned PCR products from one of the patients that acquired JAK2-V617F on both *JAK2* gene alleles. Two clones (E03 and F03) with different genotypes of the *rs12343867* single nucleotide polymorphism (arrow on right) are shown to carry the JAK2-V617F oncogenic mutation (arrow on left).

Multiple acquisitions of oncogenic mutations in the same gene have not been previously demonstrated in other hematological malignancies, but multiple *KRAS* and *KIT* mutations were identified in several types of solid tumors (191–195). Furthermore, a MPN patient that harbored the JAK2-V617F mutation as well as a deletion in exon 12 of the *JAK2* gene in two independent progenitor clones had been previously reported in literature (145). Taken together, these data serve evidence that MPN patients have an increased tendency to acquire *JAK2* mutations with a frequency higher than expected from random mutagenesis. Somatic mutations in cancer-associated genes are thought to arise from random mutagenesis resulting in equal distribution between the two parental alleles. Interestingly, we observed that the majority of patients that were heterozygous for *rs12343867* carried the JAK2-V617F mutation on the *JAK2* allele that was tagged by the "C" allele of *rs12343867* (93/109, 85%), whereas only 13 (15%) had acquired the mutation on the "T" allele (chi-square=60.38, P=7.8x10^{-15}; Figure 13C). This finding raised the hypothesis that JAK2-V617F mutations were not randomly acquired, but occurred preferentially on a particular *JAK2* allele. To test this hypothesis, we performed association analysis of the *JAK2* mutational status and the *rs12343867* genotype. The comparison of JAK2-V617F positive patients (case population, n=213) and JAK2-V617F negative patients (control population, n=120) revealed that the frequency of JAK2-V617F positive cases was higher among patients heterozygous for *rs12343867* or homozygous for the "C" allele than in cases that were homozygous for the "T" allele of *rs12343867* (Table 12, Table 13). These results served first evidence that homozygous or heterozygous carriers of the "C" allele of *rs12343867* were at higher risk of acquiring the JAK2-V617F mutation compared to carriers of the "T" allele of *JAK2*. To confirm and further elaborate these results, we performed the same association analysis using a total number of eight SNPs within the *JAK2* gene locus (Table 14). Of the eight SNPs analyzed, four showed a significant association with JAK2-V617F positive MPN (*rs3780367*, *rs10974944*, *rs12343867* and *rs1159782*; Figure 14A). These four SNPs were in linkage disequilibrium (LD) in our MPN cohort (Figure 14B). Using the genotypic data of these SNPs, two haplotypes of the genomic region (TCTT and GGCC) were predicted with frequencies of 0.50 and 0.45, respectively.

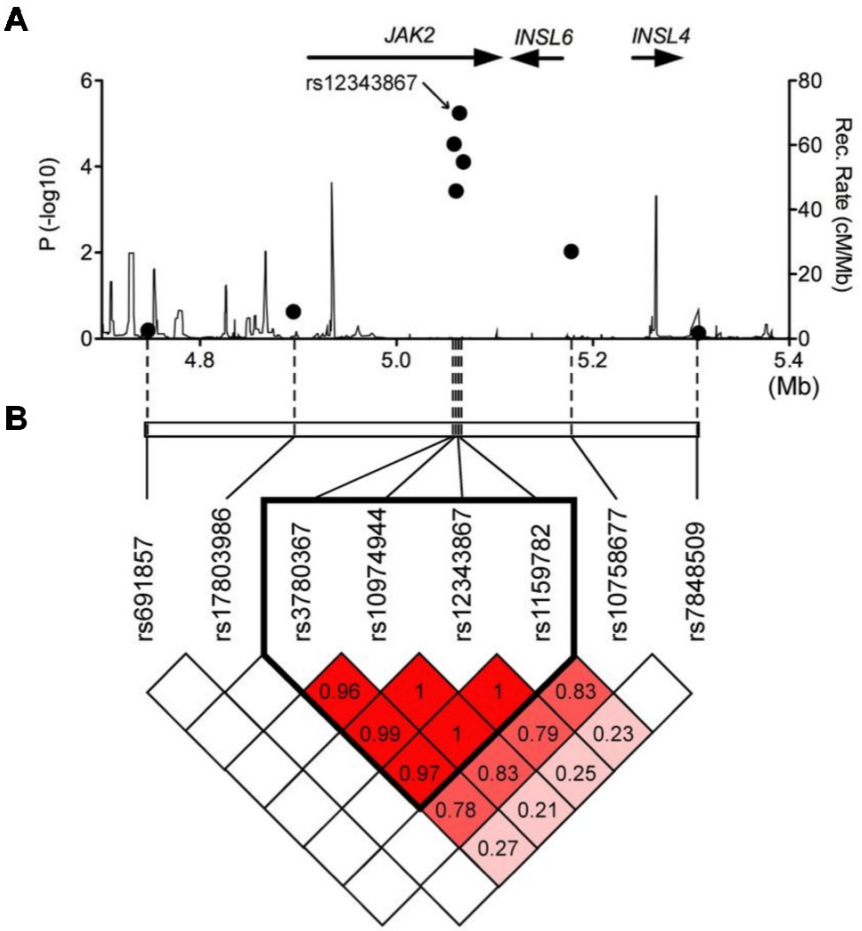


Figure 14. Association analysis of eight SNPs of the *JAK2* genomic region. A. Association analysis of SNP genotypes and JAK2-V617F mutational status of MPN patients. Chi-square test *P* values are shown on the left axis for each SNP (black dots). Chromosomal positions are based on NCBI build 36 in mega base pairs (Mb). The HapMap recombination rates (combined phase I and II, release 21) are shown on the right axis (black line). Horizontal arrows indicate Refseq gene positions (*JAK2, INSL6, INSL4*). **B.** Linkage disequilibrium (LD) structure calculated for the eight SNPs from genotypes of 335 MPN patients. Red boxes indicate LD (*D'* values shown for SNP pairs).

The results of our previous experiments served evidence that one of the two common haplotypes that harbors the *JAK2* gene preferentially acquires the JAK2-V617F mutation. To prove this hypothesis with an experimental approach, we determined the *JAK2* haplotype in MPN patients with acquired uniparental disomy of chromosome 9 (9pUPD). 9pUPD causes the transition of cells heterozygous for JAK2-V617F to homozygosity via mitotic recombination and results in the loss of the wild type *JAK2* allele (Figure 15A). Thus, patients with 9pUPD are always homozygous for JAK2-V617F and carry the haplotype that acquired the mutation on both chromosomes. First, SNP genotyping of the same eight SNPs as described

above was performed in 17 MPN patients with 9pUPD. All SNPs were homozygous and revealed the haplotypes on which the JAK2-V617F mutation had occurred in these cases (Figure 15B). The presence of 9pUPD was confirmed using Affymetrix 50K XbaI and SNP 6.0 mapping arrays showing a copy-number neutral loss of heterozygosity on chromosome 9p. Interestingly, this analysis showed that 15 of 17 MPN patients (88%) had acquired the JAK2-V617F mutation on the GGCC haplotype (Figure 15B). Subsequent association analysis comparing MPN patients with and without the JAK2-V617F mutation showed that the GGCC haplotype was more frequent in V617F positive cases (haplotype-specific chi-square=16.87, P=4x10^{-5}). Taken all together, these data indicate that a certain haplotype that harbors the *JAK2* gene, which we refer to as the GGCC haplotype, confers increased susceptibility to the acquisition of the JAK2-V617F mutation.

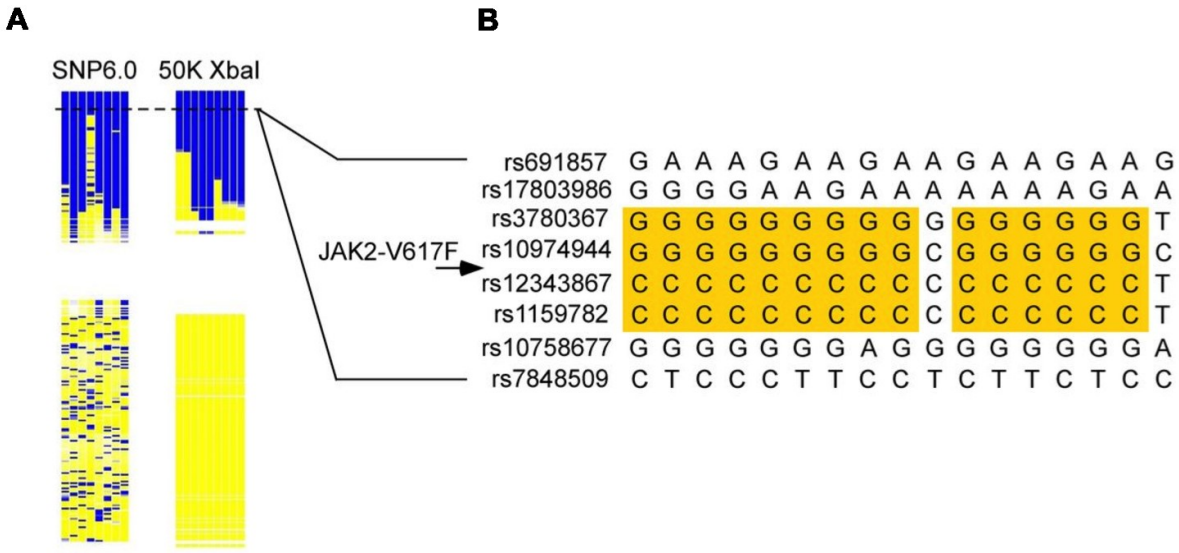


Figure 15. Determination of the *JAK2* gene haplotypes that carry JAK2-V617F in seventeen patients with uniparental disomy of chromosome 9p (9pUPD). A. Patients with 9pUPD were identified by loss of heterozygosity analysis using either the SNP 6.0 or the 50K XbaI Affymetrix microarrays. LOH (blue) was called using the dChip program (right). All patients had LOH in the *JAK2* locus and were confirmed to be homozygous for the JAK2-V617F mutation. **B.** Patients with 9pUPD carry only the haplotype that acquired JAK2-V617F. Each patient (columns) was genotyped for eight SNPs (rows). The most common haplotype (GGCC) that carries the oncogenic JAK2-V617F mutation is highlighted in color. Arrow indicates the position of the JAK2-V617F mutation relative to the analyzed SNPs.

4.2.1 *JAK2* exon 12 mutations and the *JAK2* GGCC haplotype

Initially addressing the incidence of multiple acquisitions of the JAK2-V617F mutation in MPN patients, we obtained evidence that JAK2-V617F is preferentially acquired on one of the two common *JAK2* haplotypes. This finding raised the notion that the GGCC haplotype of *JAK2* is more susceptible to DNA repair defects or replication infidelity and therefore, is more likely to acquire somatic mutations. If so, somatic mutability would not be restricted to the V617F mutation, but other genetic defects would also occur more frequently on this haplotype. To investigate this hypothesis of "differential mutability" between haplotypes, we investigated if the less frequently occurring *JAK2* exon 12 mutations also preferentially arise on the GGCC haplotype. A total number of 44 Caucasian PV patients of Western European descent that were negative for JAK2-V617F, but positive for *JAK2* exon 12 mutations were examined (Table 15). All patients were genotyped for a SNP in intron 12 of the *JAK2* gene (*rs10974944*) that is in linkage disequilibrium with the GGCC haplotype. Association analysis between the 44 *JAK2* exon 12 positive PV patients and an Austrian non-MPN control population revealed a significant correlation between the occurrence of *JAK2* exon 12 mutations and the GGCC haplotype (n=290; Table 16, Figure 16). Patients carrying the "G" variant of *rs10974944* were at higher risk of acquiring a point mutation or deletion in exon 12 of the *JAK2* gene compared to those that harbored the "C" allele (OR 2.10; 95% CI: 1.31-3.35; *P*=0.0016). Moreover, about 64% of the *JAK2* exon 12 mutation positive MPN patients were either heterozygous or homozygous for the GGCC haplotype. The minor allele frequencies did not differ significantly between the Austrian non-MPN cohort and publicly available genotype data of the Caucasian HapMap CEU and TSI populations (0.24, 0.25 and 0.30, respectively), excluding the influence of population-specific differences in SNP allele frequencies. In conclusion, these data provide evidence for differential mutability between haplotypes and indicate that increased susceptibility to somatic mutagenesis of the *JAK2* GGCC haplotype is not restricted to V617F, but also involves *JAK2* exon 12 mutations.

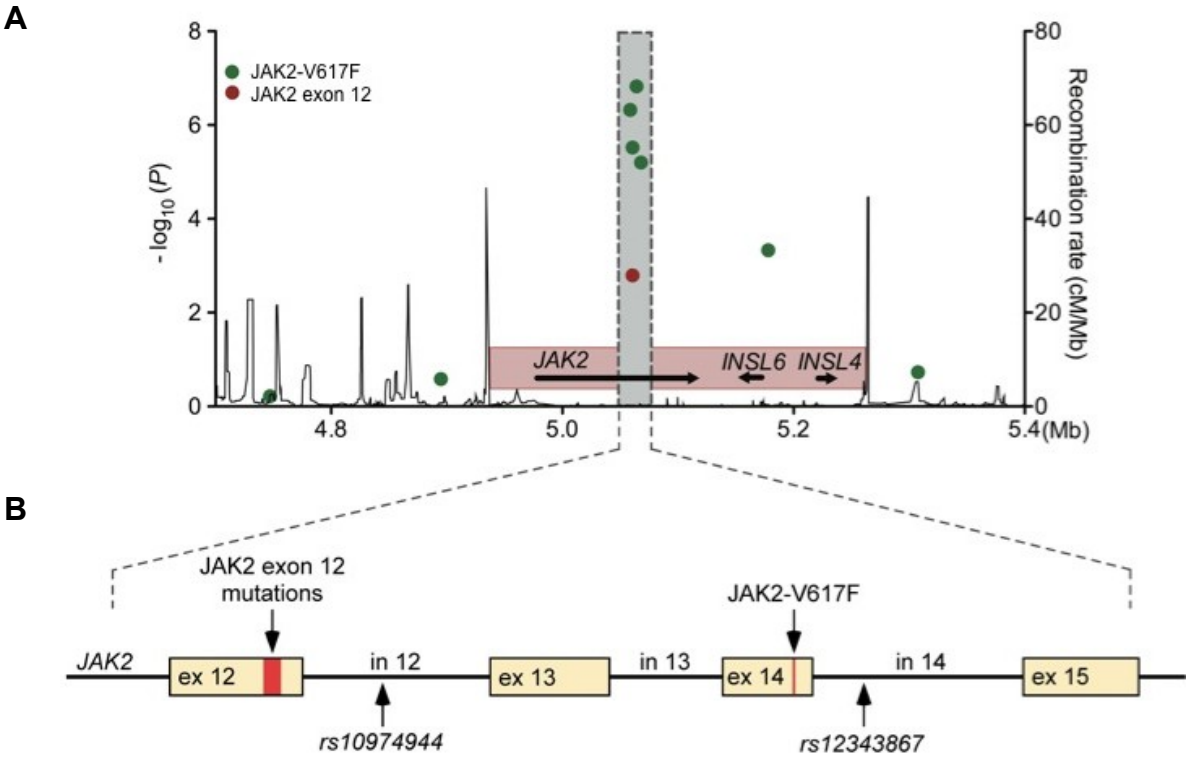


Figure 16. Association between the GGCC haplotype and *JAK2* mutations. A. Allelic association results of SNPs in the *JAK2* genomic region on chromosome 9p. Dots represent *P* values of association analysis between JAK2-V617F positive and negative MPN (green dots) and *JAK2* exon 12 mutation positive PV (red dot). The x-axis represents chromosomal position in mega base pairs (Mb). HapMap recombination rates (combined phase I and II, release 21) are shown on the right y-axis (black line). Horizontal arrows delineate the RefSeq gene position of *JAK2*, *INSL6* and *INSL4* genes. Dashed grey bar indicates the SNP-genotyped region tagging the GGCC haplotype (red horizontal bar) and containing JAK2-V617F and *JAK2* exon 12 mutations. **B.** Detailed view of the genomic region susceptible to *JAK2* mutations. *JAK2* introns (in) 11 to 15 and exons (ex) 12 to 15 are shown (not in scale). The *JAK2* exon 12 mutation locus and the SNP used for association analysis in this study (*rs10974944*) are depicted. Exon 14 and the locus of the JAK2-V617F mutation are shown with the SNP in intron 14 (*rs12343867*) that was used for association analysis.

4.3 Familial MPN

The investigation of single nucleotide polymorphisms on chromosome 9p in patients with MPN led to the identification of a certain haplotype that harbors the *JAK2* gene and confers susceptibility to somatic mutations. The finding that inherited genetic variation affects somatic mutability in MPN patients raised the question whether the identified risk haplotype, the GGCC haplotype, underlies familial clustering in MPN. In a collaboration with the Division of Hematology of the IRCCS Policlinico San Matteo, University of Pavia, Pavia, Italy, a cohort of familial MPN patients was assembled and the frequency of the *JAK2* GGCC haplotype was determined in order to elucidate the role of inherited genetic variation in the *JAK2* gene on the pathogenesis of familial MPN.

4.3.1 Patient characteristics

Interview-based investigation of familial MPN history was performed in a total number of 772 patients with apparently sporadic MPN. All investigated patients were from the area of northern Italy, resembling a demographically homogeneous population. Of the MPN patients, 88 were identified as familial cases (11%) and 684 were allocated to sporadic cases (89%). In total, 53 pedigrees with familial MPN were identified. A summary of the demographic and clinical patient characteristics of this cohort at MPN diagnosis is given in Table 17.

4.3.2 The *JAK2* GGCC haplotype in familial and sporadic MPN

To determine the role of the *JAK2* GGCC haplotype in familial clustering of MPN, we first aimed to compare the frequencies of the GGCC haplotype in familial, sporadic MPN and a non-MPN control population. In case that the *JAK2* GGCC haplotype underlies familial clustering in MPN, the frequency of the risk allele was expected to be higher in familial cases than in sporadic MPN patients. In order to compare the *JAK2* haplotype frequencies among the patient populations, the allele

frequencies of a tagging SNP of the GGCC haplotype (*rs10974944*) were used. All patients were genotyped for *rs10974944* using a TaqMan SNP genotyping assay and allelic association analysis was performed. As expected, the "G" variant of *rs10974944* that resembles the tagging variant of the *JAK2* GGCC haplotype was more frequent in familial and sporadic MPN patients (0.44 and 0.42, respectively) compared to the non-MPN control population (0.27). However, there was no significant difference in the allele frequencies of the "G" variant of *rs10974944* between familial and sporadic MPN (*P*=0.5851). Next, association analysis applying a co-dominant genotypic model was performed and showed a significant association between heterozygosity and homozygosity for the "G" variant of *rs10974944* and the JAK2-V617F mutation in both, familial and sporadic MPN cases (Table 18). The comparison of only JAK2-V617F positive cases of familial and sporadic MPN with the control population revealed a significant association between the GGCC haplotype and JAK2-V617F, whereas JAK2-V617F negative cases did not show a correlation, neither in familial nor in sporadic MPN (Table 18).

Hypothesizing that the *JAK2* GGCC haplotype underlies familial clustering in MPN, we expected a higher frequency of the haplotype in familial compared to sporadic MPN patients. Accordingly, the genotype frequencies of *rs10974944* in JAK2-V617F positive familial and sporadic MPN patients were compared. Interestingly, the results revealed no significant correlation between the GGCC haplotype and familial clustering in MPN (*P*=0.6529, Table 18). The risk of acquiring *JAK2* mutations that is conferred by the GGCC haplotype does not differ between sporadic and familial cases, regardless of the *JAK2* mutational status. Thus, the *JAK2* GGCC haplotype predisposes to the acquisition of mutations in the *JAK2* gene in familial as well as sporadic MPN, but does not underlie familial clustering.

4.3.3 Sequence analysis of *TET2*, *CBL* and *MPL*

Somatic mutations in genes that are relevant for MPN pathogenesis have been postulated as potential candidates for inherited predispositions to familial MPN. To test this hypothesis, we investigated the presence of somatic and germline mutations

in *TET2, CBL* and *MPL* genes and evaluated their role in the familial occurrence of MPN. Sequence analysis of granulocyte genomic DNA in a total of 88 affected patients with a familial history of MPN (53 pedigrees) revealed 12 cases of a non-synonymous mutation in the *TET2* gene. Two of the identified mutations were single base pair deletions or insertions causing a frame shift, whereas 7 patients carried a missense mutation (Table 19). Three patients showed a nonsense mutation resulting in a premature stop-codon, of which one had previously been described (Saint-Martin, 2009). To determine if the identified *TET2* mutations were somatically acquired or inherited in these families, we subsequently performed sequence analysis of the respective T lymphocytes DNA samples. In 5 out of 12 cases we confirmed the presence of the mutation in granulocyte and T lymphocyte DNA, whereas 7 mutations were only detectable in the granulocyte sample and thus, were somatically acquired. Interestingly, one familial MPN patient (MPC08-188) with two distinct mutations of the *TET2* gene was identified. Of the five patients with a suspected germline mutation, two carried an amino acid substitution of proline with serine at position 1723 (P1723S) and a third patient had a valine to leucine substitution at position 1718 (V1718L), all being previously described as normal variants (Table 19).

In total, two familial MPN patients with a novel *TET2* mutation (A241V and R1440Q) that was present in both, granulocyte and T lymphocyte DNA, were identified. We investigated if the other affected member of the respective MPN family carried the same mutation to evaluate the segregation of the two suspected germline variants with the MPN phenotype. There was no DNA available for molecular analysis of the other affected family member of patient MPC08-188, who harbored a germline A241V mutation and a somatic R550X mutation of *TET2*. In patient 377, the other family member with MPN did not carry any mutation of the *TET2* gene, thus excluding segregation of the R1440Q mutation with the MPN disease phenotype. Among all 53 Italian MPN pedigrees, no mutations of the *CBL* or *MPL* gene were detected. Taken together, these data imply that mutations of *TET2, CBL* and *MPL* do not account for the familial occurrence of MPN. All identified *TET2* mutations of this study are listed in Table 19.

4.3.4 Genetic heterogeneity and penetrance in familial MPN

In the frame of our genomic studies in familial MPN patients, we identified an Australian family with three members that were affected by MPN. One member of the Australian MPN pedigree was diagnosed with ET, whereas the other two affected family members were diagnosed with PMF. High-resolution genomic mapping data was obtained for all three cases using Affymetrix SNP 6.0 arrays and the *JAK2* and *MPL* mutational status was determined using standard PCR-based methods. This genetic analysis revealed a heterogeneous and partially complex mutational pattern in this particular MPN family (Figure 17A). One of the three affected members was positive for the *MPL*-W515L mutation (MPD214), whereas patient MPD227 carried the *JAK2*-V617F mutation as well as a 5.7 Mb deletion on chromosome 22q. The third evaluated member of this family was negative for *MPL* and *JAK2* mutations, but carried multiple deletions on chromosomes 2p, 7q and 15q (MPD219). Comparison of the cytogenetic and mutational status in granulocyte DNA and T lymphocyte DNA revealed that all of the identified genetic defects were somatically acquired. Furthermore, the *JAK2* haplotype was determined in all three MPN family members using TaqMan SNP genotyping of the tagging SNP *rs12343867.* The results showed that the patient who was positive for the *JAK2*-V617F mutation was heterozygous for the *JAK2* GGCC haplotype, whereas the other two affected family members were both homozygous for the non-risk haplotype (Figure 17A). Thus, neither the *JAK2* GGCC haplotype nor the other identified genetic defects did exhibit segregation with the MPN phenotype in this pedigree. Furthermore, we extended the analysis of the *JAK2* GGCC haplotype to the Italian familial MPN cohort and could show that 24 familial MPN patients did not harbor the GGCC haplotype (27.3%), 51 were heterozygous (58.0%) and 13 were homozygous carriers of the *JAK2* risk variant (14.8%) (Figure 17B, Table 18). Taken together, these data demonstrate that about one third of patients with familial MPN do not carry the *JAK2* GGCC haplotype.

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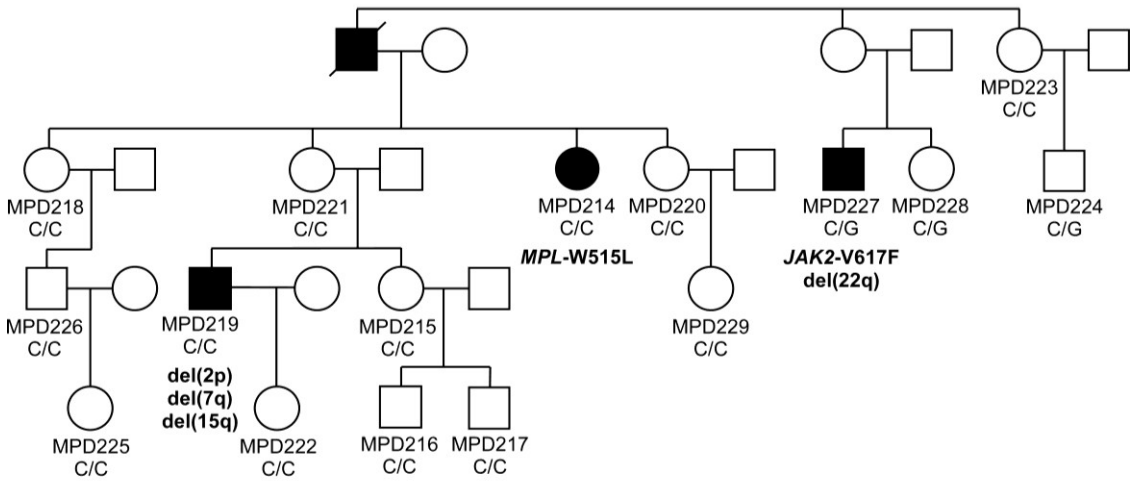

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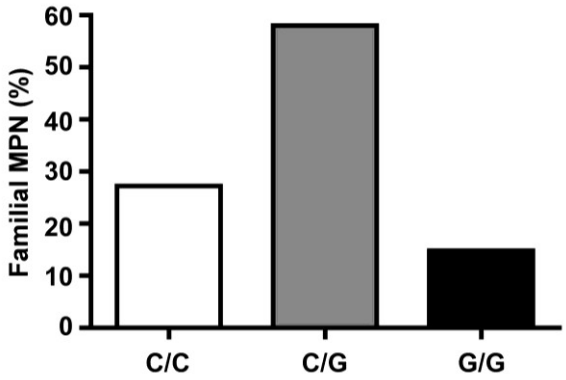

Figure 17. The *rs10974944* genotype and genetic heterogeneity in familial MPN. A. Pedigree of a MPN family with three affected members carrying distinct genetic aberrations. Family members with manifest MPN are shown as filled symbols with females depicted as circles and males as squares. Patients that are not alive are marked with a diagonal line. Individuals with DNA available for molecular analysis have assigned sample numbers (e.g. MPD221). The *rs10974944* (a tagging SNP of the *JAK2* haplotype) genotypes of all subjects with DNA available are shown. Of the three family members affected by MPN, two were homozygous for the C variant of *rs10974944* (non-risk variant of the *JAK2* haplotype) and one was heterozygous (MPD227). The familial MPN patient diagnosed with PMF was heterozygous for *rs10974944* (MPD227), positive for the *JAK2*-V617F mutation and harbored a deletion on the long arm of chromosome 22 (del(22q)). One of the other affected members was diagnosed with ET and carried the *MPL*-W515L mutation (MPD214), whereas the other affected individual (MPD219), diagnosed with PMF, had deletions on chromosomes 2p, 7q and 15q. **B.** Genotype frequencies of *rs10974944* in familial MPN. The percentages of familial MPN patients in the three genotypic classes of *rs10974944* (C/C, C/G and G/G) are shown. The G variant of *rs10974944* tags the *JAK2* GGCC haplotype, whereas the C variant represents the non-risk variant of the haplotype.

To gain further insights into the potential role of the *JAK2* GGCC haplotype in familial clustering of MPN, estimations of penetrance in familial MPN and of the GGCC haplotype were calculated and compared. The approximate penetrance in familial cases was estimated to lie within a range of 31-35%, whereas the penetrance of the *JAK2* GGCC haplotype is around 0.02%. The observed difference in penetrance illustrates that the familial predisposition to MPN is stronger than the susceptibility conferred by the *JAK2* GGCC haplotype. Thus, the *JAK2* haplotype contributes to the risk of developing MPN in familial as in sporadic cases, but is not causative in familial clustering.

4.3.5 Malignancies in familial and sporadic MPN

Genetic analysis of the Australian MPN pedigree showed that each affected family member carried different oncogenic mutations and distinct chromosomal aberrations (Figure 17A). The observed genetic heterogeneity in this MPN family suggests that the predisposition that is inherited within this pedigree does not only confer susceptibility to acquire certain somatic aberrations such as *JAK2* mutations. Thus, we hypothesized that individuals with a familial history of MPN harbor a germline genetic defect that predisposes to increased somatic mutagenesis and results in a higher incidence of various malignant disorders. To test this hypothesis, the incidence of solid tumors and hematologic malignancies in familial and sporadic MPN patients was determined and their association with the *JAK2* GGCC haplotype was evaluated. In our cohort of 772 MPN patients, 52 malignancies were recorded (6.7%), of which 21 occurred before (40%) and 31 after the diagnosis of MPN (60%). Of the 88 patients with a familial history of MPN, 10 were diagnosed with a malignancy other than MPN (11.4%), whereas only 42 of 684 sporadic MPN patients (6.1%) had an additional malignant disorder (Fisher's exact test, P=0.0721). Table 20 lists and Figure 18 graphically illustrates the frequencies of malignancies in familial and sporadic MPN patients included in this study.

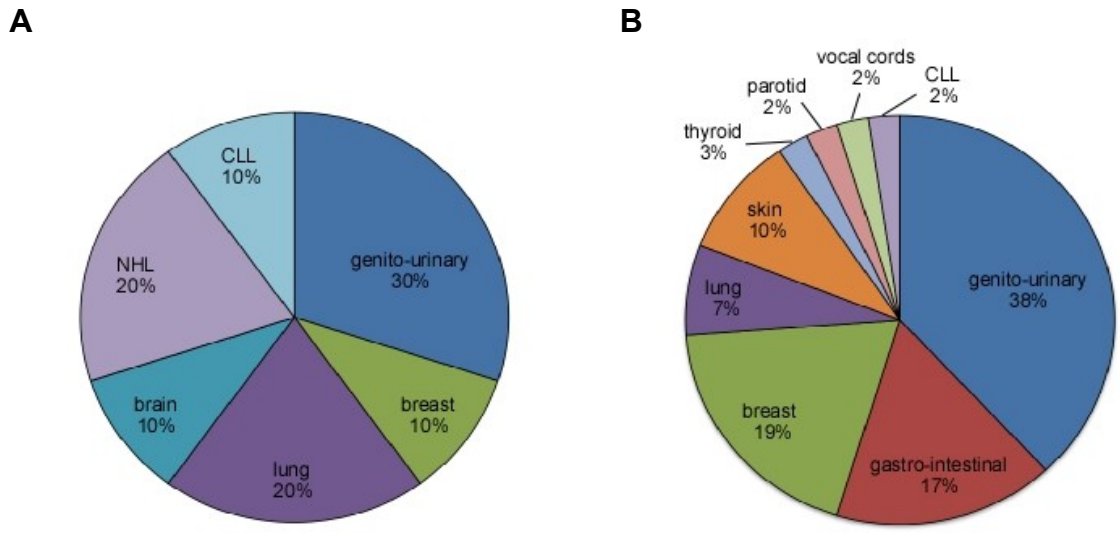

Figure 18. Malignancies in familial and sporadic MPN patients. Pie charts represent the frequencies of malignant disorders in familial and sporadic MPN. Type of cancer and frequencies in percentages are depicted for both groups. **A.** Malignancies in familial MPN patients. Lung and brain cancer as well as non-Hodgkin lymphomas (NHL) and chronic lymphocytic leukemia (CLL) are more frequent in familial MPN patients than in sporadic cases. **B.** Malignancies in sporadic MPN patients. Gastro-intestinal tumors, breast cancer and tumors of the skin occur more frequently in patients with sporadic MPN. The frequency of genito-urinary cancers does not differ significantly between familial and sporadic cases.

To evaluate the difference in the occurrence of malignancies in familial and sporadic MPN, two distinct statistical methods were applied. In the first analysis, we used a case-control model to assess the risk of developing malignancies throughout the patient's life (before MPN diagnosis and during follow-up) in familial compared to sporadic cases. Patients with familial MPN showed a by trend higher risk of developing malignancies during their life span than sporadic cases (OR 1.96, 95% CI: 0.84-4.16; *P*=0.0657). After adjustment for age at MPN diagnosis by applying 4 age categories (<40 years, 40-55 years, 55-65 years, >65 years) with similar numbers of patients in each group (Figure 19A), malignancies remained more frequent in familial MPN than in sporadic cases (OR 1.98, 95% CI: 0.94-4.18; *P*=0.06). When statistical adjustment for the presence of the *JAK2* GGCC haplotype (considering a dominant genotypic model) was applied, association analysis remained similar with an OR of 2.01 (95% CI: 0.96-4.19, *P*=0.056). Thus, we conclude that the *JAK2* haplotype does not modify the risk of developing malignant disorders in familial MPN.

In a second approach, statistical analysis was carried out based on the patient's age at last follow-up (median 58 years, range 18.7-92.2 years). MPN patients were grouped into three age categories (<50 years, 50-70 years, >70 years) with approximately equal numbers of cases in each group and the frequency of malignancies was compared between age categories in sporadic and familial MPN (Figure 19B). Although no statistically significant difference was observed, malignancies were slightly more frequent in familial than in sporadic MPN patients (OR 1.77, 95% CI: 0.84-3.71; *P*=0.1174). However, the restricted comparison of familial and sporadic MPN patients in age group 2 (50-70 years) revealed a significantly higher risk of developing malignancies in cases with a familial history of MPN (OR 3.13, 95% CI: 1.13-8.66; *P*=0.0198; Figure 19B).

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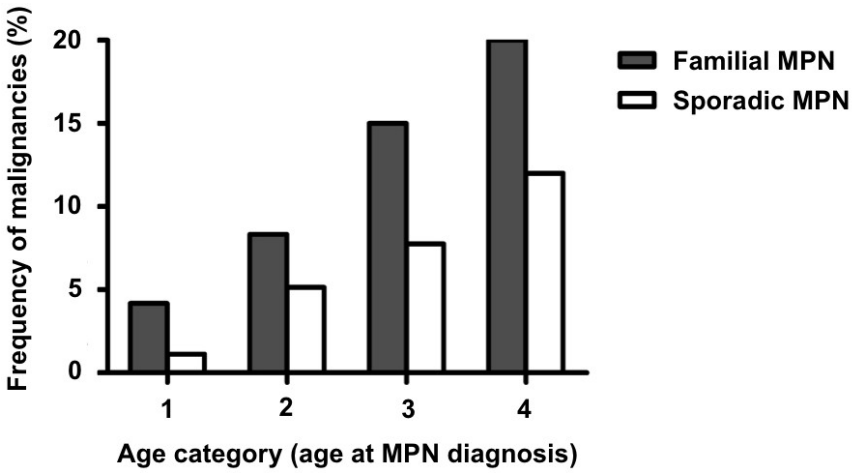

B

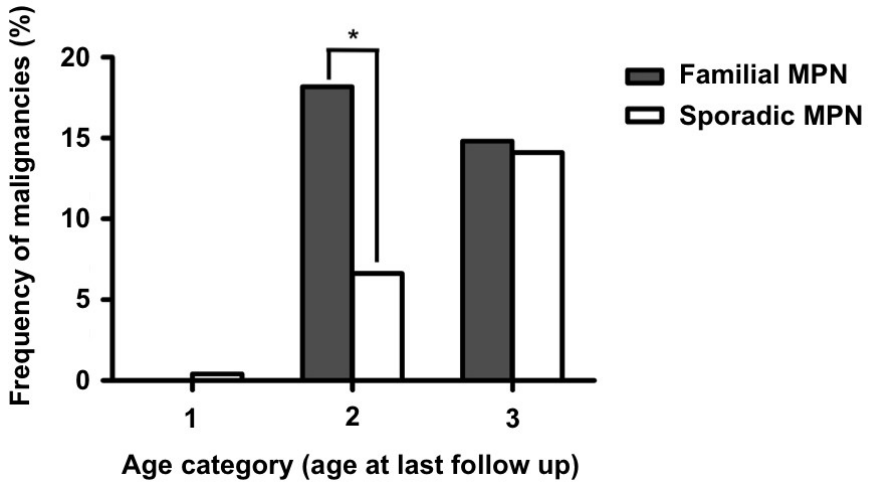

Figure 19. Frequency of malignancies in familial and sporadic MPN patients. A. Comparison of malignancies in familial and sporadic MPN. The cohort of 772 patients was grouped into 4 categories (1-4) according to the age at MPN diagnosis (<40 years, 40-55 years, 55-65 years, >65 years). Grey bars depict familial and white bars sporadic cases. In patients younger than 40 years at MPN diagnosis the frequency of malignancies was 4.17% among familial cases and 1.12% among sporadic cases (*P*=0.24); in the second age group (40-50 years) the frequency of malignancies was 8.33% among familial and 5.14% among sporadic cases (*P*=0.51); in 55-65 years-old patients the frequency of malignancies was 15% among familial cases and 7.75% among sporadic cases (*P*=0.28); in patients older than 65 years the frequency of malignancies was 20% among familial cases and 12% among sporadic cases (*P*=0.31). For each age category the frequency of malignancies in familial patients was higher than the one observed in sporadic MPN, however, not statistically significant. **B.** In a second analysis, the same patients (n=772) were grouped into 3 age categories (1: <50 years, 2: 50-70 years, 3: >70 years) according to age at last follow-up. Grey bars represent familial and white bars sporadic MPN cases. There was only one sporadic MPN patient and no familial case with a malignant disorder under the age of 50 years at last follow-up. In the second age group (50-70 years), the frequency of malignancies was significantly higher in familial patients compared to sporadic MPN cases (**P*=0.0198). There was no difference in the occurrence of malignancies in familial and sporadic MPN patients over the age of 70 years at last follow-up (*P*=0.9226).

Next, the incidence of malignancies after MPN diagnosis was compared between familial and sporadic patients. In familial MPN the incidence of cancer was 11.7 per 1000 person-years and 6.0 per 1000 person-years in sporadic MPN, with an IRR of 1.95 (*P*=0.11). Adjustment of the statistical computation for age at MPN diagnosis did not cause a significant change in the IRR (IRR 1.93), thus excluding an effect of the age at diagnosis on the incidence of malignancies in this analysis. Figure 20 shows a comparative Kaplan-Meier estimation of cancer-free survival in both patient groups. Familial MPN patients showed a trend towards shorter cancer-free survival compared to patients with sporadic MPN (logrank test, *Z*=1.4776; *P*=0.1395; Figure 20). Statistical significance was not achieved in this analysis, probably due to the overall limited number of study subjects with a familial history of MPN and a low number of tumor cases in both patient populations (7 of 88 in familial and 24 of 684 in sporadic MPN). Nevertheless, these data suggest that patients with familial clustering of MPN might harbor a yet unidentified predisposition to develop malignant disorders in general, independent from the *JAK2* haplotype.

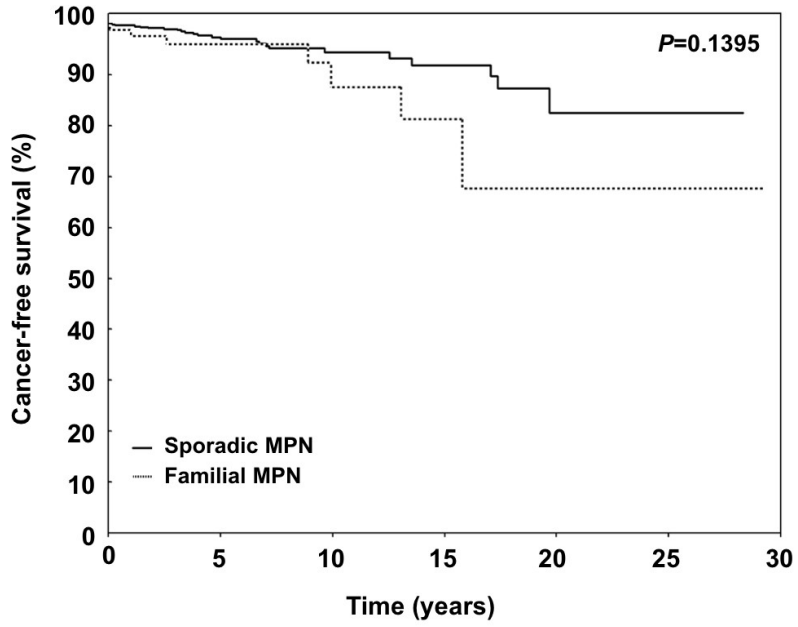

Figure 20. Cancer-free survival after MPN diagnosis in familial and sporadic MPN patients. Kaplan-Meier estimation of cancer-free survival in familial MPN (dashed line) and sporadic MPN patients (black line). The x-axis shows time after MPN diagnosis in years and the y-axis represents the percentage of patients without malignancy. Patients with a familial history of MPN have a slightly decreased cancer-free survival than sporadic MPN cases. However, there is no statistically significant difference in cancer-free survival (*P*=0.1395), most likely due to the overall low number of cancer cases in both groups (7 cancer cases among 88 patients with familial MPN and 24 cases among 684 sporadic MPN patients, respectively).

5 DISCUSSION

5.1 Chromosome 13q deletions in MPN

Myeloproliferative neoplasms represent a heterogeneous group of hematologic malignancies that share the pathogenetic feature of clonal hematopoiesis. The acquisition of genetic defects in single hematopoietic progenitor cells has been demonstrated to grant proliferative advantage and enable the evolution of a malignant disease clone. Up to date, a number of cytogenetic aberrations and mutations in various genes of important signaling and regulatory pathways have been identified and shown to trigger malignant outgrowth of aberrant hematopoietic cells. In this study, we identified deletions of chromosome 13q as recurrent genetic events in myeloproliferative neoplasms and aimed to further characterize the role of these aberrations in disease development.

Performing high-resolution genomic mapping analysis in a single-institutional MPN patient cohort we determined the frequency of del13q as being about 3% in patients with MPN. The frequency of del13q was confirmed in two additional MPN patient cohorts from Vienna, Austria, and Florence, Italy. The alignment of the deleted chromosomal region of each identified del13q case revealed a chromosomal interval that is lost in 15 out of 16 MPN patients with del13q and thus, aligns a novel common deleted region of del13q that spans about 850 kilo base pairs. Of note, only 5 out of 16 del13q cases identified in this study cover a chromosomal region of less than 5 Mb. In the remaining 11 cases with a deletion of chromosome 13q, a loss of more than 10 Mb of chromosomal material (range 10.17-45.35 Mb) was observed with all identified deletions being located between bands 13q12.3 and 13q31.1. Thus, most deletions of chromosome 13q affect a large chromosomal region and result in the heterozygous loss of a various number of genes.

Among the 7 genes identified in the novel common deleted region of del13q remains the retinoblastoma 1 (*RB1*) gene, representing the first *bona fide* tumor suppressor gene to be identified and whose mutational inactivation was shown to cause human cancer. Accordingly, *RB1* has been postulated as the target gene of chromosome 13q deletions since the first observation of del13q as a recurrent

chromosomal aberration among myeloid neoplasms (114). The retinoblastoma protein (RB) and its family members p107 and p130 mediate diverse cellular responses by controlling the activity of the E2F transcription factors and their target genes, particularly regulating the G1-S phase transition during cell cycle progression (196). The RB family members are themselves regulated by cyclin-dependent kinase (CDK) complexes, which are subjected to inhibition by the p16INK4a and p21CIP1 families. Thus, either direct inhibition of the RB family members or alterations of the above mentioned pathways that regulate their function could promote tumor formation. In fact, mutations targeting the RB pathway are common in human malignancies, however distinct cancer types are known to target different regulatory elements of this pathway (197,198).

The *RB1* gene was identified as the first tumor suppressor gene due to studies in patients with familial retinoblastoma, a pediatric tumor of the eye. At first, statistical modeling indicated that in hereditary cases of retinoblastoma one genetic alterations was inherited – the so-called “first hit” – but tumors only developed after the somatic acquisition of another mutational event, the “second hit” (199). Subsequent genetic investigations led to the identification of a deletion on chromosome 13 in retinoblastoma patients and further efforts granted the cloning of the *RB1* gene (200–205). Familial patients with retinoblastoma were shown to harbor a germline genetic defect of the *RB1* gene, whereas the retinoblastoma tumor tissue of these patients contained a deletion or mutation of the remaining *RB1* allele, leading to complete inactivation of the RB tumor suppressor in retinal cells. This second hit was a result of mitotic recombination in most subjects, whereas the some cases acquired a novel mutation in the remaining *RB1* allele. These observations led to the establishment of the “two-hit hypothesis” that served as a paradigm of hereditary cancer susceptibility, paving the way for the identification of various other tumor suppressor genes such as *TP53*, *APC* as well as *BRCA1* and *BRCA2* (206–209).

Based on previous observations on *RB1* and tumor suppressor genetics, we tested the hypothesis that *RB1* is the target gene of chromosome 13q deletions in MPN. Of note, all deletions of chromosome 13q identified in this study were hemizygous aberrations. To validate the hypothesis that mutations or microdeletions in the *RB1* gene might cause a complete inactivation of this tumor suppressor in MPN, we performed sequence analysis of the remaining *RB1* allele in patients with

del13q. Sanger sequencing of all 27 exons of *RB1* in 4 MPN patients with del13q did not reveal any mutations or microdeletions within the *RB1* gene. Similarly, no genetic aberration of *RB1* was identified in 8 del13q patients applying novel deep sequencing technologies. Thus, the remaining *RB1* allele in MPN patients with a deletion of chromosome 13q is not inactivated by a point mutation or microdeletion. These results indicate that if *RB1* is the target gene of del13q, its pathogenetic role is not impacted via complete tumor suppressor inactivation.

In case of haploinsufficiency, the loss of one gene copy is sufficient to promote a phenotype. To test the hypothesis that *RB1* is a haploinsufficient gene in MPN pathogenesis, we first performed gene expression analysis in patients with del13q. Gene expression levels in peripheral blood granulocytes did not differ significantly between four MPN patients with del13q compared to four control samples. Furthermore, no difference in gene expression was observed in CD34+ hematopoietic cells, CD34- cells and granulocytes isolated from five cord blood samples of healthy donors, indicating that *RB1* is equally expressed throughout myeloid cell differentiation. Thus, the loss of one *RB1* allele does not alter gene expression levels, most likely due to compensatory mechanisms that tightly regulate the function of this important tumor suppressor gene.

Recent reports in literature showed that the *RB1* gene is amongst the imprinted human genes (190). These investigations revealed that a differentially methylated CpG island, which serves as an alternative *RB1* promotor, is not methylated in retinoblastoma patients with a deletion of the maternal *RB1* allele, whereas it is fully methylated in cases with a deletion of the paternal allele. This finding raised the notion that chromosome 13q deletions might specifically target a certain parental allele and result in epigenetic dysregulation of *RB1* expression. To verify this hypothesis, we performed methylation-specific PCR on granulocyte and whole blood DNA of 11 MPN patients with a deletion of chromosome 13q. The results of this experiment showed variable methylation patterns with no preference towards a certain parental allele. Furthermore, no evidences for hyper- or demethylation of *RB1* in MPN patients with del13q were gained. Thus, we conclude that chromosome 13q deletions do not alter methylation-dependent epigenetic regulation of the *RB1* gene in MPN.

In order to evaluate the functional impact and cellular outcome of *RB1* deficiency in hematopoietic cells, we assayed proliferation and cytokine-sensitivity of hematopoietic cell lines and primary bone marrow cells after shRNA-mediated silencing of *RB1* gene expression. Human and murine shRNA constructs targeting *RB1* were integrated into lentiviral vectors and used to transfect human and murine hematopoietic cell lines as well as murine primary bone marrow cells. For each cell type, a proliferation assay with cultures of four to five days was conducted under increasing concentrations of cytokines. The results of these proliferation experiments showed no significant difference of proliferation and sensitivity to growth cytokines between Rb1-deficient and control cells. In conclusion, the loss of *RB1* expression does not seem to influence cytokine-sensitivity and short-term proliferation of hematopoietic cells.

Taken all together, our data suggest no obvious functional impact of the monoallelic loss of the *RB1* tumor suppressor gene in the pathogenesis of MPN. However, the functional experiments conducted in this study were limited to *in vitro* data. Previous reports in literature on mouse models indicate that the homozygous loss of *RB1* is critical for hematopoietic stem and progenitor cell function and differentiation. Firstly, mice deficient of the Rb protein are not viable and show defects in multiple tissues, including reduced formation of blood islands in the fetal liver as well as an increase of immature nucleated erythroid cells (210–212). Furthermore, investigations revealed that Rb expression levels are high during erythroid differentiation, whereas it is downregulated during the maturation of granulocytic cells (213). In our study, we did not find any significant difference in *RB1* expression between CD34+, CD34- hematopoietic cells and granulocytes from healthy controls or patients with del13q. More recent data showed that mice reconstituted with homozygous Rb-knockout fetal liver cells – containing fetal hematopoietic stem cells – developed a progressive depletion of hematopoietic cells in the bone marrow (214). In a different approach based on the constitutive deletion of Rb in hematopoietic cells, mice developed a myeloproliferation-like syndrome with an increase of myeloid cells with extramedullary hematopoiesis in the spleen, whilst the numbers of erythrocytes and B cells were reduced (215). Although peripheral blood cell counts were not increased in these mice, even after months of follow up, increased numbers of hematopoietic progenitor cells in the peripheral blood were

detected (215,216). Daria et al. could also show that Rb-deficient hematopoietic cells exhibit increased numbers of cells in cell cycle while their capacity of long-term reconstitution of the hematopoietic system is impaired (215). Compound genetic inactivation of Rb and its pocket protein family members p107 and p130 was shown to induce a loss of hematopoietic stem cell quiescence, an increase in the number of progenitor and stem cell populations, extramedullary hematopoiesis as well as an expansion of (in particular eosinophilic) granulocytes (217). These observations indicate that not only Rb but also its family members p107 and p130 are essential in hematopoiesis and that p107 and p130 hold important compensatory roles in case of Rb-deficiency. However, these studies were based on the homozygous inactivation of Rb function. Up to date, available data on the heterozygous inactivation of Rb shows that heterozygous Rb knock-out mice do not develop retinoblastomas, but about 25% of these animals show adenocarcinomas of the pituitary gland by the age of 8 to 10 months (211). Interestingly, the wild-type allele of Rb was found to be lost in these pituitary tumors, consistent with the two-hit model of tumorigenesis as proposed for Rb inactivation in retinoblastoma. Furthermore, the same study showed that mice with a constitutive homozygous inactivation of Rb were not viable after day 14.5 of gestation, most likely due to an inhibition of hepatic erythropoiesis and severe anemia. Taken all together, these data indicate a significant role of Rb in normal hematopoiesis. However, further studies are necessary in order to elucidate the impact of a heterozygous loss of Rb function in hematopoietic cells.

The newly defined common deleted region of del13q in this study harbors a total number of seven genes. As a well-known tumor suppressor gene, *RB1* was postulated the target of del13q in MPN. However, our in vitro data did not suggest a functional role of heterozygous *RB1* loss in hematopoietic cells. Sequence analysis of the six remaining del13q CDR genes did not reveal mutations or microdeletions that would result in a complete inactivation of the respective del13q gene. Based on literature search, we compiled the following data on the novel del13q CDR genes. The *SUCLA2* gene encodes the beta subunit of the succinyl-CoA synthetase (SCS-A), which represents a mitochondrial matrix enzyme of the Krebs cycle that hydrolyzes ATP to convert succinate to succinyl-CoA. Mutations in this gene account for encephalomyopathic forms of mitochondrial DNA depletion syndromes – a group of autosomal recessive disorders that are characterized by decreased mitochondrial

DNA copy numbers in the affected tissues (218–221). Major symptoms of patients with homozygous *SUCLA2* mutations are Leigh or Leigh-like syndrome with demyelinating brain lesions and cerebral atrophy, accompanied by symptoms such as high lactate levels in the blood, muscle hypotonia, deafness and renal tubular dysfunction with methylmalonic aciduria. However, all clinically manifest cases of *SUCLA2*-related mitochondrial depletion syndrome showed homozygous mutations of the *SUCLA2* gene (221).

The nudix-type motif 15 gene (*NUDT15*) is thought to encode an 8-oxo-dGTP diphosphatase that belongs to the superfamily of nudix hydrolases. Members of the nudix (nucleoside diphosphate linked moiety X) hydrolases superfamily are believed to hydrolize nucleoside diphosphates linked to different moieties. Their function is to repair oxidatively damaged nucleosides and nucleotides, serving as regulators of different cell cycle stages and stress periods. Up to date, a number of different nudix hydrolases has been identified, although the exact function of most members of this superfamily remains to be elucidated. The protein encoded by *NUDT15* (also known as MutT Homolog 2, *MTH2*) has been shown to degrade 8-hydroxy-dGTP in vitro (222). Furthermore, 8-hydroxy-dGTP was shown to specifically induce mutations due to A:T to C:G transversions in live simian and human cells (223,224). Recent literature reports that an siRNA-mediated knockdown of the NUDT15 protein in the human 293T tumor cell line increases the number of A:T to C:G substitution mutations induced by 8-hydroxy-dGTP (225). These data support the role of nudix hydrolases as nucleotide pool sanitization enzymes and suggest that mutations in these genes might establish a mutator phenotype. However, further research and evidence is needed to support these hypotheses.

The mediator complex subunit 4 (*MED4*) gene is known to encode a coactivator protein that is required for activation of RNA polymerase II by a variety of DNA-binding transcription factors and has been implicated in vitamin D signaling as a part of the vitamin D3 receptor-interacting protein complex (DRIP36). No mutations in this gene have been reported in literature. A study investigating genotype-phenotype correlations in retinoblastoma patients with interstitial chromosome 13q deletions reported that patients with 13q deletions larger than 1 Mb and including the *MED4* gene showed a milder retinoblastoma phenotype compared to patients with small 13q deletions (226). The mechanism by which this phenotypic difference is

established was suggested to relate to the function of *MED4* in vitamin D signaling, but the details of this association remain unclear.

The integral membrane protein 2B gene (*ITM2B*) encodes a transmembrane protein that is further processed by furin and furin-like proteases and results in a small peptide that inhibits the deposition of beta-amyloid in various tissues. Mutations in this gene have been identified to result in an elongation of the secreted peptide at the C-terminus, leading to increased deposition of amyloid as observed in neurodegenerative diseases such as familial British and familial Danish dementia (227). Recently, a peptide of this protein, Bri2-23, has been implicated in cerebellar dysfunction of patients with multiple sclerosis and was proposed as a potential biomarker (228). However, mutations in *ITM2B* have not been reported to affect hematopoietic cells.

The *RCBTB2* gene encodes a member of the regulator of chromosome condensation (RCC1) family that serves as a guanine nucleotide exchange factor (GEF) for the Ras-related GTPase Ran. RCC1 proteins are known to convert GDP to GTP and play an important role in the control of cell cycle progression through the Ran signal transduction pathway (229). Furthermore, loss of RCC1 was shown to alter nuclear protein export and deteriorate cell cycle checkpoint control (230). A report in literature identified loss of heterozygosity at chromosome 13q14 in about 50% of prostate cancer samples and suggested *RCBTB2* as the target gene of 13q14 LOH, although further investigation could not support a role for *RCBTB2* loss in prostate cancer (231). Also, a number of studies investigating the role of *RB1* alterations in prostate cancer failed to reveal any association (232,233).

The lysophosphatidic acid receptor 6 (*LPAR6*) gene encodes a G-protein coupled receptor that is activated by adenosine and uridine nucleotides. *LPAR6* is expressed in the inner root hair sheath of the hair follicle and is important for the normal development of the hair shaft. Homozygous mutations of this gene as well as its ligand, lysophosphatidic acid, are associated with familial autosomal recessive woolly hair (ARWH) syndromes (234). A role for *LPAR6* mutations in hematopoietic disorders is not known up to date.

Although none of the novel del13q CDR genes has been implicated in hematopoietic cell function, we cannot exclude a role of these genes in the

pathogenesis of del13q in MPN. In fact, inclusion of the *MED4* gene in the deleted region of chromosome 13q in retinoblastoma patients has been associated with a milder disease phenotype, whereas alterations of *RCBTB2* and *NUDT15* have been suggested to play a role in mutagenesis. Thus, further experimental data is needed in order to define the impact of a monoallelic loss of these genes in hematopoietic cell function and to identify the target gene of del13q in MPN.

Chromosome 13q deletions are the most frequent chromosomal aberration in patients with chronic lymphocytic leukemia (CLL), being present in about 50% of cases (235). Early investigations identified several chromosomal regions affected by 13q deletions, some of which result in the loss of *RB1* (119). Thus, it was first suggested that *RB1* might be the pathogenetic effector of del13q in CLL. Further studies revealed a high frequency of deletions at a more telomeric locus on chromosome 13q amongst multiple non-overlapping common deleted regions, indicating that several tumor suppressor loci might be involved in the evolution of clonality in CLL (121,236–239). After the identification of the *miR15a/16-1* microRNA cluster as the target of del13q in CLL, *in vivo* experiments in mouse models demonstrated that the loss of microRNAs is sufficient to establish a lymphoproliferative phenotype (122,240). Interestingly, these experiments showed that mice with a deletion of the minimal deleted region including the *DLEU2* gene developed a more aggressive disease than mice with a sole knockout of the *miR-15a/16-1* gene cluster. Supporting evidence for a multigenic mechanism of del13q in CLL was gained by the observation of different types of 13q deletions in CLL patients, either affecting only the microRNA cluster, including the mono- or biallelic loss of *RB1*, or targeting a larger genomic region (116). Deletions on chromosome 13q including the *RB1* locus were found to associate with elevated genomic complexity in CLL (241). A recent study showed that large 13q deletions of more than 2 Mb size at the time of CLL diagnosis are associated with an increased risk of disease progression and that this association is independent of other prognostic factors (242). These findings emphasize that although minimally deleted regions serve an important tool to identify target genes of recurrent chromosomal deletions, they might oversimplify the role of various deletion sizes and heterogeneous effects of multigenic loss in cancer pathogenesis. In this study, we observed that close to 70% (n=11/16) of del13q events affected a chromosomal region of more than 10 Mb,

resulting in a monoallelic loss of a large number of genes. No single gene deletions were identified and the smallest chromosome 13q deletion contained seven genes. Thus, a multigenic mechanism based on a collaboration of multiple tumor suppressor and/or oncogene loci could be postulated for del13q in MPN. However, the frequency of del13q is significantly higher in CLL than in MPN and the identification of further del13q cases might reveal a different distribution of del13q sizes in MPN. Of note, 13 out of 16 patients with del13q in our MPN cohort carried a deletion that contains the *RB1* locus as well as the *miR-15a/16-1* microRNA cluster. As suggested by the above-mentioned studies for CLL, the loss of both loci might be equally associated with an aggravated phenotype in MPN.

In this study, hemizygous deletions of chromosome 13q were identified as recurrent genetic aberrations in MPN that affect about 3% of patients. Genotypic analysis of hematopoietic progenitor cell colonies showed that del13q is not the genetic founder lesion in MPN, as it can occur before or after other recurrent chromosomal defects. Furthermore, we could refine the minimally deleted region of chromosome 13q to a genetic locus containing seven genes. Complete inactivation via a mutation or deletion of these genes in patients with del13q could be excluded through further analysis. Thus, we primarily suggest haploinsufficiency within this chromosomal region as the underlying genetic mechanism of clonal proliferation in del13q positive hematopoietic cells. However, in the majority of del13q cases the deleted region on chromosome 13q encompasses a genomic region of more than 10 Mb and harbors a varying number of genes. Several previous studies of recurrent deletions in other hematologic neoplasms have suggested a multigenic origin of malignant disease pathogenesis. Accordingly, varying deletion sizes of chromosome 13q deletions and the subsequent loss of various genes within this region might influence the resultant disease phenotype in del13q positive MPN. Further elaboration of these hypotheses will grant important insights into genetic mechanisms not only in MPN, but will deepen our understanding of the genetic complexity of cancer pathogenesis in general.

5.2 The *JAK2* GGCC haplotype

The genotypic analysis of hematopoietic progenitor cell colonies for genetic aberrations such as deletions of chromosome 13q and the JAK2-V617F mutation led to the identification of a patient with two independent JAK2-V617F positive hematopoietic cell clones. To test the hypothesis that multiple acquisitions of JAK2-V617F represent recurrent genetic events in MPN, we developed a PCR-based assay and could show that about 3% of MPN patients acquire the JAK2-V617F mutation in at least two independent hematopoietic cell clones. Furthermore, we observed an unequal distribution of JAK2-V617F mutational events between the two *JAK2* alleles. We confirmed this finding by genotype analysis of single nucleotide polymorphisms (SNPs) in linkage disequilibrium and furthermore, identified a common *JAK2* haplotype that preferentially acquires the oncogenic JAK2-V617F mutation. Our analysis revealed that more than 80% of JAK2-V617F mutations in MPN patients occur on the *JAK2* GGCC haplotype. Although the nature of this association remains unclear, there are currently two alternative hypotheses: (i) *JAK2* mutations occur randomly on both haplotypes, but the GGCC haplotype holds certain features or "*cis* regulatory elements" necessary to propagate an MPN disease phenotype, or (ii) the GGCC haplotype is more susceptible to somatic mutagenesis and thus, the V617F mutation preferentially arises on this particular sequence variant of the *JAK2* gene.

A common hypothesis is that haplotypes contribute to disease by the alteration of gene expression or by a specific modification of protein function through non-synonymous SNPs. Although the GGCC haplotype contains the promoter region of the *JAK2* gene*,* two of the genotyped SNPs in this study that lie within this region are not in linkage disequilibrium with the GGCC haplotype. Thus, it is unlikely that the GGCC haplotype holds influence on *JAK2* gene expression via the promotor region. Based on a search in public databases, we found that the SNPs within the GGCC haplotype are not in linkage disequilibrium with any non-synonymous SNPs that might influence protein function of the mutant *JAK2* kinase. In fact, MPN is not confined to patients carrying the GGCC haplotype or cases with a JAK2-V617F mutation and thus, MPN development cannot be restricted to cases where the JAK2-V617F mutation is acquired in a certain sequence context. We identified three MPN patients with multiple JAK2-V617F acquisitions in this study. In two cases, the clone

that had acquired the JAK2-V617F mutation on the TCTT haplotype predominated the second clone that carried the JAK2-V617F on the GGCC haplotype. This observation suggests that the GGCC haplotype does not hold substantial properties that are required by the V617F mutation to confer proliferative advantage.

Previous reports in literature have shown that DNA sequence variations in the *APC* and *TP53* genes predispose these loci to somatic mutagenesis (243,244). The obtained data in this study raises the hypothesis that DNA sequence variants might define somatic mutability and render haplotypes more susceptible to DNA damage or replication infidelity. Thus, differential mutability between haplotypes might underlie the preferential occurrence of the JAK2-V617F mutation on the GGCC haplotype. To further elaborate this hypothesis, we aimed to elucidate whether the GGCC haplotype confers susceptibility to mutations other than V617F. Thus, we performed association analysis between *JAK2* exon 12 mutation-positive MPN cases and the GGCC haplotype. The obtained data confirmed that not only V617F mutations, but also exon 12 mutations of *JAK2* preferentially occur on the GGCC haplotype, serving further evidence for the comparably increased somatic mutability of this *JAK2* sequence variant. A recent meta-analysis of several studies in MPN patients suggested an association between the *JAK2* GGCC haplotype and mutations of the *MPL* gene in MPN patients, thus providing evidence for a regulatory effect of the GGCC haplotype (245). However, two subsequent studies could not confirm these data, implicating that the acquisition of *JAK2* and *MPL* mutations is based on different genetic mechanisms (246,247).

The concept of “differential mutability” as a haplotype property has not been considered in investigations of cancer susceptibilities so far. Nevertheless, our data suggest that haplotypes influence genetic repair or replication mechanisms and establish susceptibility as well as an accumulation of defects at that particular genomic region. Still, this study does not serve a mechanistic prove for this hypothesis and we cannot exclude the possibility that the GGCC haplotype is in linkage with a yet unidentified functional variant that influences MPN disease manifestation and outcome. From a general perspective, previously identified cancer susceptibility loci could represent genomic regions of differential mutagenesis and thus, might carry somatic mutations of relevance for cancer pathogenesis. The identification of genomic loci with similar properties as the *JAK2* GGCC haplotype

could serve a proof of principle for the postulated difference in somatic mutability of haplotypes and support the notion, that similar genetic factors might influence cancerogenesis in other malignant diseases.

5.3 Familial MPN

The *JAK2* haplotype identified in this study is a common genetic variant and was shown to represent an inherited disease predisposition that possibly influences mutability at this locus. As such, it was suggested as a germline genetic factor that might underlie familial clustering of MPN. To define the role of the *JAK2* GGCC haplotype in familial MPN, we performed genotype analysis in a cohort of patients with a familial history of MPN. Based on the hypothesis that the *JAK2* GGCC haplotype underlies familial clustering in MPN, an increased frequency of the risk haplotype was expected in familial compared to sporadic MPN cases. However, the results of our association analysis showed no significant correlation between the *JAK2* GGCC haplotype and the acquisition of JAK2-V617F when familial and sporadic cases were compared. Thus, the *JAK2* GGCC haplotype frequency and the risk of acquiring a mutation at that particular locus do not differ between familial and sporadic MPN and the *JAK2* GGCC haplotype does not underlie familial clustering of MPN. As in cases with sporadic MPN, the GGCC haplotype was more frequent in familial MPN than in the control population, confirming an association between the haplotype and the acquisition of *JAK2* mutations. Genotypic analysis revealed that about 30% of familial MPN patients do not carry the *JAK2* GGCC haplotype and the estimated penetrance in familial MPN was about three orders of magnitude higher than the penetrance of the *JAK2* GGCC haplotype. These data together with previous reports that excluded linkage of the *JAK2* gene locus with familial PV serve further evidence that the *JAK2* GGCC haplotype confers susceptibility to *JAK2* mutation-positive MPN, but does not explain familial clustering (55).

Following a different approach to identify inherited genetic factors that underlie familial clustering in MPN, we performed sequence analysis of the *TET2*, *CBL* and *MPL* gene. These genes have been implicated in MPN disease pathogenesis and

were hypothesized to represent candidates for inherited predisposition to familial MPN. Mutations of the *MPL* gene have been identified in familial as well as sporadic cases of ET, whereas only one case of a germline *TET2* mutation has been reported up to date (62,248,249). The *CBL* gene has been implicated in cases with juvenile myelomonocytic leukemia (250). Thus, sequence analysis of *TET2*, *CBL* and *MPL* was performed in a cohort of 88 patients with familial MPN. Although several acquired mutations of the *TET2* gene were found, no mutations of these genes that are inherited and segregate with the disease phenotype were identified. Thus, germline mutations of *TET2*, *CBL* and *MPL* do not explain familial clustering of MPN and the inherited genetic defect that underlies familial MPN remains yet to be identified.

Previous reports in literature served evidence for disease anticipation in familial MPN by identifying an early disease onset and significant telomere shortening in second-generation MPN patients (251). Additionally, a study with more than 24,000 first-degree relatives of about 11,000 MPN patients revealed an increased risk of developing MPN, other chronic hematologic malignancies such as CML and CLL or solid tumors such as malignant melanoma and brain cancer among relatives of MPN patients (252). These observations nurtured the idea that a general inherited predisposition to familial MPN might exist, which is not restricted to the development of MPN but establishes increased overall tumorigenesis. In this study, we identified an MPN pedigree with different genetic aberrations in each of the affected family members as well as distinct manifestations of the MPN phenotype, suggesting a common disease predisposition that causes various genetic lesions and different phenotypic outcomes, respectively. In order to further elaborate the hypothesis that patients with familial MPN exhibit a general increase in somatic mutability, we evaluated the incidence of malignancies in our cohort of 53 MPN pedigrees. The incidence of malignant disorders was higher in familial than in sporadic MPN patients, particularly in patients between the age of 50 to 70 years at last follow-up. In fact, we could show that familial MPN patients have a 3-fold increased risk of developing malignancies other than MPN. The observed types of cancer and their frequencies were different between familial and sporadic MPN. For example, tumors of the gastro-intestinal tract and skin were more frequent in sporadic MPN, whereas no patient with familial MPN showed these types of cancer. On the other hand, Non-

Hodgkin lymphomas were more frequent in familial than in sporadic MPN patients and CLL as well as brain tumors were only identified in familial cases. Kaplan-Meier estimates of cancer-free survival in familial and sporadic MPN patients showed – although statistically not significant – a decrease in cancer-free survival in familial cases. Taken all together, our data support the hypothesis of a higher risk for malignant disorders in patients with familial MPN. The number of familial cases was limited in this study, due to the fact that familial MPN is a rare disorder. Still, larger patient cohorts will be necessary to further characterize inherited disease predispositions and to identify the genetic defect that underlies familial clustering in MPN.

Based on the results of this study, we hypothesize that there is a mutation – of e.g. a tumor suppressor gene – that establishes increased somatic mutability and enables the consecutive acquisition of various genetic defects in familial MPN. Previous investigations have shown that mutations or deletions of certain tumor suppressor genes, such as *TP53*, *VHL*, *APC*, *BRCA* and *CDKN2A*, exhibit specific genotype-phenotype correlations (253–257). The identification of the *JAK2* GGCC haplotype as well as the notion of increased cancer incidence in familial MPN patients demonstrates that germline genetic factors influence somatic mutagenesis and promote various cancer phenotypes. Further examinations on the genetic mechanisms behind cancer predisposition in MPN and other malignant diseases will improve our understanding of the interplay between inherited and acquired genetic factors in cancer evolution.

TABLES

Table 1. JAK2-V617F allele-specific PCR primers

Primer	Sequence (5'-3')	5' dye	Primer mix
JAK2V617F-F	GTTTCTTAGTGCATCTTTATTATGGCAGA	None	4μM
JAK2V617F-RG	TTACTCTCGTCTCCACAGAC	6-FAM	2μM
JAK2V617F-RT	AAATTACTCTCGTCTCCACAGAA	6-FAM	2μM

Table 2. General PCR master mix

Master Mix*	1x	10x	100x
10x PCR GOLD Buffer	1.1	11	110
25mM $MgCl_2$	0.66	6.6	66
2.5mM dNTPs	0.44	4.4	44
4μM Primer mix	1.4	14	140
AmpliTaq GOLD (5U/μL)	0.05	0.5	5
DNA template (10ng/μL)	1	-	-
HPLC-H_2O	6.36	63.6	636
Total volume	11.01μl	100.1μl	1001μl

Table 3. Microsatellite PCR primers

Marker	Forward primer (5'-3')	Reverse primer (5'-3')	5' dye
D13S153	GACTCCTGTTTCTCCTCCCTG	ATTTGTGGAAAGGAGCGTAT	5-HEX
D13S263	CCTGGCCTGTTAGTTTTTATTGTTA	GTTTCTTCCCAGTCTTGGGTATGTTTTTA	6-FAM
D13S1233	AGTTCCTAGGGGCAACACTA	GTTTCTTGACGTGGGATGTCATAGAG	6-FAM
CEMM13S1	TTGGACTTATAACAGCTGGC	GTTTCTTAAATTTTGTTTCCAGCTCTG	5-HEX
CEMM13S2	CAGCCTCCTAAGTACCCTCC	GTTTCTTGTAATCCTCCTGCCTCCTGG	6-FAM
CEMM13S4	CCTGTGCATTCACATTTTGT	GTTTCTTCAGTGAGCTGTGATTGCACC	5-HEX
CEMM13S5	TTGGGTATTGTTTCACATTGAG	GTTTCTTCAGTTGTTTCCAGTTTTTCAG	5-HEX
CEMM13S6	CCGCTCCTCTGTGTTTGTG	GTTTCTTGCACGAGGAGAGGTGGC	6-FAM
CEMM13S7	ATTTGCTGGTGTCCAAGAGG	GTTTCTTAAAAGCCCGTTTTCAGAAGG	5-HEX
CEMM13S8	ACCTCGAGTCCACCCTCCTG	GTTTCTTCGCCACCCCTCTTCATCTTG	6-FAM
CEMM13S9	AATGGCAACTACAACTTGCAC	GTTTCTTGTGCTACTACACCCCAGCC	6-FAM
CEMM13S10	GCTCCTAAGCTTGCAGATGG	GTTTCTTGAGGAACCGAACTAACGGAAT	6-FAM
CEMM13S11	GGGAGATGGGTTAACTGATATGAA	GTTTCTTAATGCATGGAAAGGAGGACA	6-FAM
CEMM13S12	CCCGGTCTTTACAATGGAGA	GTTTCTTTGACATCTGGGTTGGTCTGA	6-FAM

Table 4. TaqMan gene expression assays

Gene	Assay number	Species
RB1	Hs01078066_m1	Human
HPRT1	Hs99999909_m1	Human
Rb1	Mm00485586_m1	Murine
Hprt1	Mm01545399_m1	Murine
Itm2b	Mm01545399_m1	Murine
Lpar6	Mm00613058_s1	Murine
Med4	Mm00482779_m1	Murine
Rcbtb2	Mm00506375_m1	Murine
Sucla2	Mm01310541_m1	Murine

Table 5. Primers for sequence analysis of the *RB1* gene

Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')	Size (bp)
Exon1	TTTTGTAACGGGAGTCGGG	GTCACCATTCTGCAGACGC	414
Exon2	TGGTATCCTTATTTTGGAATGACC	CATGAACCACCGTGCCC	524
Exon3	TGCCATCAGAAGGATGTGTTAC	TGGCAGTTCACTATTTGGTCC	411
Exon4	TGTAGAGCTGATAATCTTTTGAATTG	AATTTTCCAGGAAGCATTCAG	423
Exon5	TTGGGAAAATCTACTTGAACTTTG	CACAGGACTTAAATCTATGGGC	337
Exon6	AAATTATGCAATTAAAATGGACTGC	CCAAGCAGAGAATGAGGGAG	348
Exon7	ACCATGCTGATAGTGATTGTTG	ATGGGCAAAGTCCATGTCTG	360
Exon8	GCAGAGTAGAAGAGGGATGGC	TGATTCCAGAGTGAGGGAGC	558
Exon9	TTGACACCTCTAACTTACCCTGC	TTTCACCACAATTCTACTTGGC	318
Exon10	TTTATATTGCATGCGAACTCAG	GGTAACTGTTATAGGACACACAATTC	469
Exon11	TAAGCAGCAGCTGGGTCATC	CACCACACCTGGCCTTC	371
Exon12	GAGACAAGTGGGAGGCAGTG	AAGCAAGAAAAGATTATGGATAACTAC	344
Exon13	TGCTTATGTTCAGTAGTTGTGGTTAC	TAGCAGCATACACAGGCAGC	420
Exon14	GGGCAAAACAGTGAGACTCC	CTTGATGCCTTGACCTCCTG	266
Exon15-16	CTGGCAACAGAGCAAGACAC	TTCTCCTTAACCTCACACTATCCC	411
Exon17	GGTTTAACCTTTCTACTGTTTTCTTTG	TCCCTATATGTTCTTGAGGTAGATG	407
Exon18	ATTGTCAATTGGGAATTTCG	TGCAAATCCTAGGTGATTCAG	504
Exon19	TGATGATGACAAGCAGTTTTCC	AATAGTCAAGAAAGATCTCGCAAC	498
Exon20	GAAAATCCATGCCCCTTCTC	CCTAGCTACCTGGGAGGC	663
Exon21	TTTGTTCTTTAAACACACTTTGGG	CATAATTACCCTTATCTTTCCAATTC	486
Exon22-23	TCCTTTATAATATGTGCTTCTTACCAG	GCAAATAGAGTTTCAAGAGTCTAGC	635
Exon24	TGTCAGTGGTTCTAGGGTAGAGG	ATGCCTGGATGAGGTGTTTG	280
Exon25	AAATGAAGTTATTACCTTTGCCTG	CTTGGCATGAAAGAAATTGG	362
Exon26	CATAAAGTAAGTCATCGAAAGCATC	GCATAAACAAACCTGCCAAC	260
Exon27	GCCATCAGTTTGACATGAGC	TGGCCATAAACAGAACCTGG	266

Table 6. Del13q breakpoint PCR primers

Primer	Forward primer (5'-3')	Reverse primer (5'-3')	Size (bp)
Pair1	GAGAGGAGACCAGCCAAGAA	CCAAATGATGCTCTTTGCTTA	326
Pair2	GGTTGGGCTGTGGAAATGT	ACCAAATGATGCTCTTTGCTTA	240
Pair3	TCAAGTTAGGAGTTGAGGAAGTTG	TTCTGCACTGTAAATTATCCATGA	328

Table 7. Methylation-specific PCR primers

Primer	Sequence (5'-3')	Annealing	5' dye
RB1-MF	GGTTTCGTTTTTTATGGTCGGGTACGGTTTACG	58°C	none
RB1-MR	AAAAACGTAAAAACGACGACCATACC	58°C	6-FAM
RB1-UF	GGTTTTGTTTTTTATGGTTGGGTATGGTTTATG	58°C	none
RB1-UR	CATAAAAACAACAACCATACC	58°C	6-FAM

Table 8. Target sequences of shRNA constructs

Construct	Species	Target gene	Sequences (5'-3')
RB1	Human	RB1	CAGAGATCGTGTATTGAGATT
Rb1_1	Mouse	Rb1	AATCCTGGAAGGATATAT
Rb1_2	Mouse	Rb1	ATGGAATCCATGCTTAAA
Random	Human	none	TAACAGGCTAGAATGTTTT
Random_1	Mouse	none	GGACGGAACAAACATCGCGCG
Random_2	Mouse	none	ACATGTTGCACGGCTCAGGAT

Table 9. Primers for sequence analysis of the *TET2*, *CBL* and *MPL* genes

Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')	Size
TET2			**(bp)**
Exon3_1	CAGTTTGCTATGTCTAGGTATTCCG	AGGCCCACTGCAGTTATGTG	698
Exon3_2	TGAACCTTCTCTCTCTGGGC	GTCTGTGCGGAATTGATCTG	540
Exon3_3	CACACATGGTGAACTCCTGG	AAGCAATTGTGATGGTGGTG	556
Exon3_4	TCTGTTCAGGTTCCAGCAG	TGCTGGCAGTTGTCCTGTAG	538
Exon3_5	GCCTCAGAATAATTGTGTGAACAG	TTTTGGAACTGGAGATGTTGG	528
Exon3_6	AAATTCCAACATGCCTGGG	TTCACCATGAAAACATTCTTCC	544
Exon3_7	TCCCAGAGTTCACATCTCCC	AGTTGCGCAGCTTGTTGAC	545
Exon3_8	TTTTGCAGGAAACAAGACCC	AAACTGCTTCAGATGCTGCTC	547
Exon3_9	TTAAGGTGGAACCTGGATGC	AGCCTTTACAAATTGCTGCC	588
Exon4	GCCCTTAATGTGTAGTTGGGG	ACAATTGTTTACTGCTTTGTGTG	283
Exon5	AACCGTTCATTTCTCAGGATG	GGCATGAGTCTTTGATCTGG	317
Exon6	CATGCTGATAAATGTTGCCC	ATAAATGTAAAAGTGCACGCTG	450
Exon7	TCAATTGAAATAGTTCTGTGTGTGG	AAAATAGTGTGTATCTACAGTTTGGG	448
Exon8	GGGATTCAAAATGTAAGGGG	TGCAGTGGTTTCAACAATTAAG	323
Exon9	TGTCATTCCATTTTGTTTCTGG	CCATGTGTTTTGGGAAGGAC	606
Exon10	CTAGGCCACCAACACAAATC	CAGAACTTACAAGTTGATGGGG	561
Exon11_1	CGTATATCACTAGTGGAGTTTCTTACC	ACAGATCCATCGGCTGAGAC	551
Exon11_2	TCTAATCCCATGAACCCTTACC	CCTTGTTTTGGAGATGCAGG	523
Exon11_3	GATGGCCACTTCATGGGAG	ATGCTGGTAAAAGACGAGGG	525
Exon11_4	GCTTTCTGGATCCTGACATTG	ACTGTGACCTTTCCCCACTG	550
CBL			
Exon_8	AGGACCCAGACTAGATGCTTTC	GGCCACCCCTTGTATCAGTA	387
Exon_9	CTGGCTTTTGGGGTTAGGTT	TCGTTAAGTGTTTTACGGCTTT	400
MPL			
Exon_10	AGAGTAGGGGCTGGCTGG	GTTTACAGGCCTTCGGCTC	232

Table 10. Summary of the loss of heterozygosity screening data in MPN

Marker	***D13S1233***	***D13S263***	***CEMM13S1***	***CEMM13S4***	***D13S153***	***rs9535032***	***CEMM13S2***	***CEMM13S7***	***CEMM13S8***	***CEMM13S9***	***rs9535416***
Chr:13q	39975098	40978921	41201887	47727312	47788735	47910016	47965341	48244312	48809470	49185372	49521891
P10	2	2	U	2	2	U	2	2	2	2	U
P26	0	U	U	X	0	U	X	0	1	0	U
P52	0	0	0	0	1	1	1	0	0	0	U
P59	U	0	0	1	0	0	1	U	0	0	0
P60	0	0	0	0	1	0	0	0	0	0	0
P64	U	1	U	0	0	0	0	0	0	0	U
P66	U	U	U	0	1	0	0	U	0	0	U
P82	U	0	U	0	1	U	0	0	0	0	U
P116	0	0	0	X	X	1	U	0	U	X	1
P123	U	X	X	U	X	U	X	1	1	U	0
P130	U	X	X	2	X	2	1	1	1	1	1
P133	X	0	X	0	X	1	0	0	0	0	U
P145	U	0	0	U	X	U	X	1	0	X	U
P154	U	0	0	0	1	U	0	U	0	0	0
P183	2	2	U	2	2	X	2	2	2	2	U
P213	U	X	0	U	1	U	U	0	U	0	0
P218	U	1	U	1	1	U	0	1	1	U	U
P220	U	1	1	U	X	U	U	1	1	1	U
P230	U	U	U	X	U	U	U	U	1	U	X
P342	0	0	U	0	1	0	X	0	U	0	U
P354	U	U	U	0	0	1	U	0	U	0	U
P366	0	U	U	U	U	X	U	U	U	U	1
P444	U	0	0	1	0	0	U	1	U	0	U

Chr:13q, physical position on chromosome 13q in base pairs; 0, no LOH; 1, partial LOH; 2, full LOH; X, no data available.

Table 11. Summary of MPN patients with del13q

Patient	Diagnosis	JAK2-V617F	*JAK2* exon 12	MPL-W515L	Other aberrations
P10	PMF	-	-	-	14qUPD, gain 3q
P130	PV	+	-	-	9pUPD
P146	PMF	-	-	+	1pUPD, del20q
P183	PMF	+	-	-	-
P214	PV, sMF	+	-	-	9pUPD
P218	PMF	+	-	-	gain 9p, del18p
P220	PV, sMF	+	-	-	9pUPD
P230	PV	+	-	-	9pUPD
P366	PMF	-	-	-	del4q
P444	PMF, sAML	+	-	-	9pUPD, del4q, del7p
P448	PV	+	-	-	9pUPD, del12q
P522	PMF	+	na	na	del2p
P672	PMF	-	na	na	-
F427	PMF	+	na	na	9pUPD, del20q
F430	PMF	+	na	na	9pUPD
F460	PV, sMF	+	na	na	9pUPD, del20q

P, Austrian MPN patient cohort; F, Firenzian MPN patient cohort; PV, polycythemia vera; PMF, primary myelofibrosis; sMF, secondary myelofibrosis; sAML, secondary acute myeloid leukemia; na, not available; UPD, uniparental disomy; del, deletion.

Table 12. Genotype-specific association of SNPs from the *JAK2* locus on chromosome 9 and MPN

Case	Control	SNP	*P* [a]	*P* [b]	Genotypes			Odds ratio (95% CI)		
JAK2-V617F positive MPN (*n* = 213)	*JAK2*-V617F negative MPN (*n* = 120)	rs691857	0.6365	0.3517	AA	AG	GG	1	1.21 (0.74–1.96)	1.32 (0.65–2.70)
		rs17803986	0.2366	0.3775	GG	GA	AA	1	0.85 (0.51–1.42)	1.44 (0.75–2.77)
		rs3780367	2.99×10^{-5}	5.23×10^{-6}	TT	TG	GG	1	2.18 (1.28–3.74)	4.99 (2.42–10.31)
		rs10974944	0.0003712	0.0002067	CC	CG	GG	1	2.50 (1.48–4.23)	3.46 (1.65–7.27)
		rs12343867	5.69×10^{-6}	9.57×10^{-7}	TT	TC	CC	1	2.36 (1.37–4.06)	5.73 (2.75–11.92)
		rs1159782	7.78×10^{-5}	2.75×10^{-5}	TT	TC	CC	1	2.56 (1.50–4.40)	4.29 (2.05–8.98)
		rs10758677	0.009399	0.00489	AA	AG	GG	1	1.10 (0.66–1.82)	2.26 (1.07–4.79)
		rs7848509	0.7354	0.4573	CC	CT	TT	1	1.18 (0.71–1.97)	1.25 (0.66–2.39)
JAK2-V617F positive MPN (*n* = 213)	Non-MPN Austrian (*n* = 99)	rs691857	0.5815	0.8804	AA	AG	GG	1	0.80 (0.48–1.34)	1.11 (0.51–2.44)
		rs17803986	0.2173	0.1878	GG	GA	AA	1	0.97 (0.56–1.66)	1.72 (0.84–3.49)
		rs3780367	2.36×10^{-9}	5.49×10^{-10}	TT	TG	GG	1	3.43 (1.97–5.98)	11.09 (4.56–27.00)
		rs10974944	2.21×10^{-9}	4.25×10^{-9}	CC	CG	GG	1	4.44 (2.57–7.69)	8.00 (3.25–19.71)
		rs12343867	1.47×10^{-10}	5.33×10^{-11}	TT	TC	CC	1	3.97 (2.26–6.99)	12.76 (5.21–31.26)
		rs1159782	1.07×10^{-8}	6.03×10^{-9}	TT	TC	CC	1	3.87 (2.21–6.77)	9.14 (3.71–22.56)
		rs10758677	0.000141	2.64×10^{-5}	AA	AG	GG	1	2.10 (1.18–3.73)	4.52 (2.16–9.46)
		rs7848509	0.2096	0.132	CC	CT	TT	1	1.07 (0.63–1.82)	1.92 (0.90–4.12)
MPN all (*n* = 333)	Non-MPN Austrian (*n* = 99)	rs691857	0.4456	0.6178	AA	AG	GG	1	0.74 (0.46–1.21)	1.01 (0.48–2.13)
		rs17803986	0.4219	0.2706	GG	GA	AA	1	1.03 (0.62–1.70)	1.53 (0.77–3.01)
		rs3780367	2.33×10^{-6}	4.81×10^{-7}	TT	TG	GG	1	2.42 (1.49–3.96)	6.28 (2.68–14.71)
		rs10974944	1.04×10^{-6}	8.28×10^{-7}	CC	CG	GG	1	3.02 (1.85–4.91)	4.95 (2.10–11.69)
		rs12343867	3.89×10^{-7}	1.03×10^{-7}	TT	TC	CC	1	2.68 (1.64–4.39)	6.82 (2.91–16.00)
		rs1159782	8.93×10^{-6}	2.82×10^{-6}	TT	TC	CC	1	2.56 (1.56–4.19)	5.25 (2.22–12.39)
		rs10758677	0.001285	0.0003669	AA	AG	GG	1	2.02 (1.21–3.52)	3.39 (1.73–6.64)
		rs7848509	0.2386	0.1893	CC	CT	TT	1	1.00 (0.61–1.64)	1.77 (0.86–3.64)

MPN, myeloproliferative neoplasms; [a]Chi square test; [b]Cochran-Armitage trend test.

Table 13. Allelic association of SNPs from the *JAK2* locus on chromosome 9 and MPN

Case	Control	SNP	Position (bp)	*P*	Alleles	Allelic odds ratio (95% CI)
JAK2-V617F positive MPN (n=213)	JAK2-V617F negative MPN (n=120)	rs691857	4747094	0.3247	A / G	1.19 (0.84-1.67)
		rs17803986	4895038	0.3634	G / A	1.16 (0.84-1.6)
		rs3780367	5058755	7.17×10^{-6}	T / G	2.12 (1.52-2.94)
		rs10974944	5060831	0.0006708	C / G	1.75 (1.27-2.43)
		rs12343867	5064189	2.13×10^{-6}	T / C	2.18 (1.58-3.02)
		rs1159782	5068117	7.64×10^{-5}	T / C	1.94 (1.39-2.69)
		rs10758677	5178078	0.006383	A / G	1.58 (1.14-2.19)
		rs7848509	5307534	0.4489	C / T	1.13 (0.82-1.57)
JAK2-V617F positive MPN (n=213)	Non-MPN Austrian (n=99)	rs691857	4747094	0.8762	A / G	0.97 (0.68-1.39)
		rs17803986	4895038	0.1717	G / A	1.27 (0.9-1.8)
		rs3780367	5058755	4.11×10^{-10}	T / G	3.15 (2.18-4.55)
		rs10974944	5060831	3.24×10^{-8}	C / G	2.78 (1.92-4.01)
		rs12343867	5064189	6.44×10^{-11}	T / C	3.31 (2.29-4.78)
		rs1159782	5068117	2.44×10^{-8}	T / C	2.8 (1.94-4.05)
		rs10758677	5178078	2.32×10^{-5}	A / G	2.1 (1.49-2.98)
		rs7848509	5307534	0.1335	C / T	1.31 (0.92-1.85)
MPN all (n=333)	Non-MPN Austrian (n=99)	rs691857	4747094	0.6028	A / G	0.91 (0.65-1.28)
		rs17803986	4895038	0.2608	G / A	1.21 (0.87-1.67)
		rs3780367	5058755	4.77×10^{-7}	T / G	2.41 (1.7-3.41)
		rs10974944	5060831	3.01×10^{-6}	C / G	2.28 (1.6-3.23)
		rs12343867	5064189	1.49×10^{-7}	T / C	2.5 (1.76-3.54)
		rs1159782	5068117	6.30×10^{-6}	T / C	2.21 (1.56-3.14)
		rs10758677	5178078	0.0004677	A / G	1.77 (1.28-2.45)
		rs7848509	5307534	0.1872	C / T	1.25 (0.9-1.73)

MPN, myeloproliferative neoplasms; bp, base pairs; *P*, Chi-square test; CI, confidence interval.

Table 14. Allele and genotype frequencies of 8 SNPs from the *JAK2* gene locus

SNP	Population	*n*	Allele	Allele frequency	Genotypes	Genotype frequency
rs691857	Non-MPN Austrian	99	A / G	0.656 / 0.344	AA / AG / GG	0.427 / 0.458 / 0.115
	MPN all	333		0.676 / 0.324		0.483 / 0.386 / 0.131
	JAK2-V617F(+) MPN	213		0.663 / 0.337		0.464 / 0.397 / 0.139
	JAK2-V617F(-) MPN	120		0.7 / 0.3		0.517 / 0.367 / 0.117
rs17803986	Non-MPN Austrian	99	G / A	0.598 / 0.402	GG / GA / AA	0.351 / 0.495 / 0.155
	MPN all	333		0.552 / 0.448		0.32 / 0.465 / 0.215
	JAK2-V617F(+) MPN	213		0.539 / 0.461		0.32 / 0.437 / 0.243
	JAK2-V617F(-) MPN	120		0.576 / 0.424		0.319 / 0.513 / 0.168
rs3780367	Non-MPN Austrian	99	T / G	0.719 / 0.281	TT / TG / GG	0.51 / 0.418 / 0.071
	MPN all	333		0.516 / 0.484		0.259 / 0.514 / 0.227
	JAK2-V617F(+) MPN	213		0.449 / 0.551		0.186 / 0.525 / 0.289
	JAK2-V617F(-) MPN	120		0.632 / 0.368		0.385 / 0.496 / 0.12
rs10974944	Non-MPN Austrian	99	C / G	0.73 / 0.27	CC / CG / GG	0.531 / 0.398 / 0.071
	MPN all	333		0.542 / 0.458		0.255 / 0.576 / 0.17
	JAK2-V617F(+) MPN	213		0.493 / 0.507		0.185 / 0.616 / 0.199
	JAK2-V617F(-) MPN	120		0.63 / 0.37		0.378 / 0.504 / 0.118
rs12343867	Non-MPN Austrian	99	T / C	0.716 / 0.284	TT / TC / CC	0.505 / 0.423 / 0.072
	MPN all	333		0.503 / 0.497		0.237 / 0.532 / 0.231
	JAK2-V617F(+) MPN	213		0.433 / 0.567		0.163 / 0.541 / 0.297
	JAK2-V617F(-) MPN	120		0.625 / 0.375		0.367 / 0.517 / 0.117
rs1159782	Non-MPN Austrian	99	T / C	0.714 / 0.286	TT / TC / CC	0.5 / 0.427 / 0.073
	MPN all	333		0.53 / 0.47		0.253 / 0.553 / 0.194
	JAK2-V617F(+) MPN	213		0.47 / 0.53		0.177 / 0.586 / 0.236
	JAK2-V617F(-) MPN	120		0.632 / 0.368		0.385 / 0.496 / 0.12
rs10758677	Non-MPN Austrian	99	A / G	0.5 / 0.5	AA / AG / GG	0.245 / 0.51 / 0.245
	MPN all	333		0.361 / 0.639		0.116 / 0.489 / 0.394
	JAK2-V617F(+) MPN	213		0.322 / 0.678		0.101 / 0.442 / 0.457
	JAK2-V617F(-) MPN	120		0.429 / 0.571		0.143 / 0.571 / 0.286
rs7848509	Non-MPN Austrian	99	C / T	0.621 / 0.379	CC / CT / TT	0.364 / 0.515 / 0.121
	MPN all	333		0.568 / 0.432		0.332 / 0.472 / 0.196
	JAK2-V617F(+) MPN	213		0.557 / 0.443		0.317 / 0.48 / 0.203
	JAK2-V617F(-) MPN	120		0.588 / 0.413		0.358 / 0.458 / 0.183

MPN, myeloproliferative neoplasms; *n*, number of cases.

Table 15. Summary of *JAK2* exon 12 mutation-positive PV patients

Location	ID	*JAK2* exon 12 mutation
Vienna/Austria	PV01	E543-D544del
	PV02	R541K, E543-D544del
Basel/Switzerland	PV03	ins33
	PV04	I540-E543delinsMK
	PV05	F537-K539delinsL
	PV06	I540-E543delinsMK
	PV07	N542-E543del
	PV08	H538-K539delinsL
	PV09	N542-E543del
	PV10	N542-E543del
	PV11	N542-E543del
	PV12	I540-E543delinsMK
	PV13	F537-K539delinsL
	PV14	E543-D544del
	PV15	E543-D544del
	PV16	K539L
	PV17	K539L
Pavia/Italy	PV18	N542-E543del
	PV19	E543-D544del
	PV20	N542-E543del
	PV21	R541-E543delinsK
	PV22	E543-D544del
	PV23	V536-I546dup11
	PV24	N542-E543del
	PV25	F537-I546dup10, F547L
	PV26	N542-E543del
	PV27	R541-E543delinsK
Dijon/France	PV28	H538QK539L
	PV29	R541-E543delinsK
	PV30	F537-I546dup10, F547L
	PV31	K539L
	PV32	F537-I546dup10, F547L
	PV33	F537-I546dup10, F547L
Bordeaux/France	PV34	N542-E543del
	PV35	N542-E543del
	PV36	K539L
	PV37	F537IK539L
Salt Lake City/USA	PV38	K539L
	PV39	N542-E543del
	PV40	F537-K539delinsK
	PV41	I540S, R541-E543delinsK
Florence/Italy	PV42	F537-K539
	PV43	K539L
	PV44	F547V

PV, polycythemia vera; del, deletion; ins, insertion; dup, duplication.

Table 16. Association analysis of *rs10974944* and *JAK2* exon 12 mutation positive PV

Model	Allele / Genotype			Chi-square	*P* value	Odds ratio (95% CI)		
Allelic	C	G		9.908	0.001646	1	2.10 (1.313-3.348)	
Trend	C	G		10.130	0.001456	1	2.10 (1.313-3.348)	
Dominant	CC	CG/GG		6.538	0.01056	1	2.32 (1.20-4.48)	
Recessive	CC/CG	GG		7.763	0.01494	1	3.68 (1.39-9.70)	
Genotypic	CC	CG	GG	11.160	0.00378	1	1.96 (0.98-3.93)	5.09 (1.80-14.45)

Trend, Cochran-Armitage trend test; CI, confidence interval.

Table 17. Characteristics of 772 MPN patients at diagnosis

	Familial MPN	Sporadic MPN	Total
No. (%)	**88 (11)**	**684 (89)**	**772**
Diagnosis, No. (%)			
PV	36 (41)	222 (32)	258
ET	36 (41)	364 (53)	400
PMF	14 (16)	64 (9)	78
Post-PV MF	1 (1)	20 (3)	21
Post-ET MF	0 (0)	12 (2)	12
Unclassifiable MPN	0 (0)	2 (1)	2
CML	1 (1)	0	1
Sex, No. (%)			
Male	42 (48)	295 (43)	337 (44)
Female	46 (52)	389 (57)	435 (56)
Age, years			
Median	52.9	51	51.4
Range	16.2-77.8	13-85.7	13-85.7
Thrombosis, No. (%)			
Before diagnosis	7 (7.9)	68 (9.9)	75 (9.7)
At diagnosis	7 (7.9)	41 (5.9)	48 (6.2)
Hemorrhage, No. (%)			
Before diagnosis	1 (1.1)	25 (3.6)	26 (3.3)
At diagnosis	1 (1.1)	8 (1.1)	9 (1.1)

ET, essential thrombocythemia; CML, chronic myeloid leukemia; MF, myelofibrosis; MPN, myeloproliferative neoplasms; PMF, primary myelofibrosis; PV, polycythemia vera.

Table 18. Genotype association analysis of *rs10974944* and *JAK2*-V617F

Case	Control	Genotype frequency (%) *case population*			Genotype frequency (%) *control population*			Odds ratio (95% CI)			*P* value
		CC	GC	GG	CC	GC	GG	CC	GC	GG	
familial MPN (n=88)	control (n=203)	24 (27.3)	51 (58.0)	13 (14.8)	114 (56.2)	69 (34.0)	20 (9.9)	1	4.36 (2.18-8.7)	4.77 (1.9-11.99)	1.193×10^{-05}
familial MPN V617F+ (n=61)	control (n=203)	13 (21.3)	37 (60.7)	11 (18.0)	114 (56.2)	69 (34.0)	20 (9.9)	1	4.8 (2.38-9.67)	4.78 (1.88-12.2)	4.929×10^{-06}
familial MPN V617F- (n=27)	control (n=203)	11 (40.7)	14 (51.9)	2 (7.4)	114 (56.2)	69 (34.0)	20 (9.9)	1	2.10 (0.90-4.89)	1.04 (0.21-5.03)	0.2042
sporadic MPN (n=684)	control (n=203)	223 (32.6)	353 (51.6)	108 (15.8)	114 (56.2)	69 (34.0)	20 (9.9)	1	2.97 (2.21-4.00)	3.73 (2.42-5.76)	3.27×10^{-15}
sporadic MPN V617F+ (n=481)	control (n=203)	125 (26.0)	268 (55.7)	88 (18.3)	114 (56.2)	69 (34.0)	20 (9.9)	1	3.54 (2.45-5.11)	4.01 (2.32-6.94)	7.19×10^{-13}
sporadic MPN V617F- (n=202)	control (n=203)	98 (48.5)	84 (41.6)	20 (9.9)	114 (56.2)	69 (34.0)	20 (9.9)	1	1.42 (0.93-2.15)	1.16 (0.59-2.29)	0.2620
familial MPN V617F+ (n=61)	sporadic MPN V617F+ (n=481)	13 (21.3)	37 (60.7)	11 (18.0)	125 (26.0)	268 (55.7)	88 (18.3)	1	1.33 (0.68-2.59)	1.2 (0.51-2.81)	0.6975

Table 19. Summary of *TET2* mutations in 88 familial MPN patients

Patient	Diagnosis	*TET2* exon	Variant	Granulocytes	T lymphocytes	Amino acid change	UniProt	References
MPC08-188	Post-ET MF	3	C/T	+	+	A241V	na	na
377	PMF	10	G/A	+	+	R1440Q	na	na
F1P1	PV	11	C/T	+	+	P1723S	VAR_058192	(161, 258)
F17P1	PV	11	G/T	+	+	V1718L	VAR_058190	(161, 259)
F20P2	PV	11	C/T	+	+	P1723S	VAR_058192	(161, 258)
MPC08-188	Post-ET MF	3	C/T	+	-	R550X	na	(61)
MaA	PV	3	C/T	+	-	Q743X	na	na
F6P4b	PV	3	del A	+	-	Frameshift	na	na
MPC08-12	PV	7	G/A	+	-	G1275R	na	na
MPC08-22	PV	9	ins T	+	-	Frameshift	na	na
SM	PMF	9	G/A	+	-	D1376N	na	na
MPC07-371	PV	10	G/T	+	-	E1483X	na	na

Abreviations: ins, insertion; del, deletion; na, not available.

Table 20. Occurrence of malignancies in familial and sporadic MPN

Site of cancer	Familial MPN	Sporadic MPN	Total
No. (%)	**88 (11)**	**684 (89)**	**772 (100)**
Before diagnosis, No. (%)	3 (3.4)	18 (2.6)	21 (2.7)
Genito-urinary	2	6	8
Gastro-intestinal	0	1	1
Breast	0	5	5
Lung	0	1	1
Skin	0	2	2
NHL	1	0	1
Thyroid	0	1	1
Parotid	0	1	1
Vocal cords	0	1	1
Post-diagnosis, No. (%)	7 (7.9)	24 (3.5)	31 (4)
Genito-urinary	1	10	11
Gastro-intestinal	0	6	6
Breast	1	3	4
Lung	2	2	4
Brain	1	0	1
Skin	0	2	2
CLL	1	0	1
NHL	1	1	2

CLL, chronic lymphocytic leukemia; NHL, Non-Hodgkin Lymphoma.

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CURRICULUM VITAE

Personal Information

Name:	**Dr. med. Damla Olcaydu Kraucher**
Date/place of birth:	April 20, 1982 in Vienna, Austria
Citizenship:	Austria

Education and Degrees

Since 08/2011	Specialty training in hematology (FMH Hämatologie) at the Department of Hematology of the University Hospital Zurich in Zurich, Switzerland
2008 – 2011	Ph.D. studies at the Medical University of Vienna and the Research Center for Molecular Medicine (Ce-M-M-), Austrian Academy of Sciences, Vienna, Austria
April 18, 2008	M.D. degree, graduation with the dissertation entitled "*Genetic Basis of Thrombosis in Essential Thrombocythemia*"
2000 – 2008	Studies of Medicine at the Medical University of Vienna, Austria
1992 – 2000	General-educative secondary school (BRG XV., Henriettenplatz 6, 1150 Vienna), graduation with GPA 1,1
1988 – 1992	Elementary school (Oskar-Spiel-Schule, 1150 Vienna)

Scholarships

DOC-fFORTE scholarship of the Austrian Academy of Sciences for the Ph.D. project entitled *"The Role of Chromosome 13q Deletions in Myeloproliferative Neoplasms".*

"Förderstipendium" of the Medical University of Vienna for the dissertation with the title *"Genetic Basis of Thrombosis in Essential Thrombocythemia".*

Prizes and Awards

"Researcher of the Month October 2010" of the Medical University of Vienna

First prize for the best presentation at the "6th International Medical Postgraduates Conference" in Hradec-Kralove, Czech Republic; November 2009

Wilhelm Türk-Prize 2009 of the Austrian Society for Hematology and Oncology (OeGHO) for the publication *"A common JAK2 haplotype confers susceptibility to myeloproliferative neoplasms"* in *Nature Genetics* in March 2009; September 2009

"Best Poster" award at the PhD-Symposium of the Young Scientists Association (YSA) of the Medical University of Vienna for the poster *"A common JAK2 haplotype confers susceptibility to JAK2 mutations in myeloproliferative neoplasms"*; June 2009

Thesis Award 2009 of the "Verein zur Förderung von Wissenschaft und Forschung" for the thesis work entitled *"Genetic basis of thrombosis in essential thrombocythemia"*; June 2009

Invited Lectures

OeGHO Frühjahrstagung 2010, Bregenz, Austria, April 2010
Oral presentation with the title *"The molecular pathogenesis of MPN and MDS"*

Languages

German, English and Turkish	fluent in spoken and written
French	moderate
Spanish	basic

Personal Interests

Music (playing the violin and accordion, solo and in ensemble/orchestra), opera and concerts, literature, photography, cinema, sports (swimming, jogging, dancing)

Details on Medical Education

Since August 2011	Specialty training in hematology (FMH Hämatologie) at the University Hospital Zurich in Zurich, Switzerland
18. April 2008	Graduation from the Medical University of Vienna, Austria, with the dissertation entitled *"Genetic Basis of Thrombosis in Essential Thrombocythemia"*
October 2007	Final medical exam
2000 – 2008	Studies of Medicine at the Medical University of Vienna, Austria

Elective Courses

2004 – 2006	Tutor in "Applied Hospital Hygiene" elective course of the Clinical Institute of Hygiene and Medical Microbiology (Vienna General Hospital, Univ.-Prof. Dr. Walter Koller)
February/March 2003	Elective course "Scientific Working" at the Institute of Medical Physiology (Vascular Biology and Thrombosis Research, Univ.-Prof. Dr. Bernd R. Binder)
May – December 2002	Tutor in Anatomy-Dissection-Course ("Anatomia Practica", Institute of Human Anatomy, Univ.-Prof. Dr. Wilhelm Firbas)

Medical Clerkships

September 2005	Charité – University Medicine Berlin, Germany; 4 weeks of internal medicine with main focus on hematology and oncology
July 2005	Medical Faculty of the Istanbul University, Turkey; 4 weeks of internal and emergency medicine
February 2004	Hospital of Melk, Melk, Lower Austria, Austria; 2 weeks of internal medicine
August 2003	Medical University of Cerrahpasa, Istanbul, Turkey; 4 weeks of accident surgery

Other Educational Activities

February 2005	IPOKRaTES-Seminar „Signs, Symptoms and Interpretation in Internal Medicine“ in Vienna, KH Rudolfsstiftung Lecturer: Steven L Mackey, MD, PhD, Columbia University, New York
September 2003	IPOKRaTES-Seminar „Cardio-Respiratory Physiology“ in Prague, Czech Republic Lecturers: Richard M. Schwartzstein MD, Harvard Medical School, Boston; Harold L. Manning MD, Dartmouth Medical School; James C. Leiter MD, Dartmouth-Hitchcock Med Center, New Hampshire.

Details on Scientific Training

April 2008 – June 2011

Ph.D. studies at the Medical University of Vienna and the Research Center for Molecular Medicine (Ce-M-M-), Austrian Academy of Sciences:

Research work focused on cancer genomics in hematologic malignancies, conducting projects on somatically acquired aberrations and inherited genetic factors in the pathogenesis of myeloproliferative neoplasms

Supervisor: Robert Kralovics, Ph.D.

September 2006 – April 2008

Research assistant and dissertation student at the Scientific Laboratory for Hematology, Medical University of Vienna, Department of Internal Medicine I, Division of Hematology, Vienna General Hospital (AKH Wien):

Research work on the genetic basis of thrombosis in myeloproliferative neoplasms, investigating the influence of genetic variation on clinical complications in myeloproliferative neoplasms

Supervisors: Univ.-Prof. Dr. Heinz Gisslinger; Robert Kralovics, Ph.D.

April 2003 – July 2006

Research assistant at the Department of Vascular Biology and Thrombosis Research (Institute of Medical Physiology, Univ.-Prof. Dr. Bernd R. Binder)
Supervisor: Univ.-Prof. Dr. Johannes Breuss

Selected Scientific Meeting Attendances

61st Meeting of Nobel Laureates in Lindau, Germany, July 2011. *Nominated delegate of the Medical University of Vienna.*

EuroScience Open Forum 2010, Torino, Italy, July 2010. *Nominated delegate of the Austrian Academy of Sciences.*

Congress of the European Hematology Association, Barcelona, Spain, June 2010. *Poster presentation (Abstract number 410).*

COST Action BM0902 *MPN*/MPNr-EuroNet First *Workshop, Nantes, France, April 2010. Poster presentation.*

American Society of Hematology 50th Annual Meeting, San Francisco CA, USA, December 2008. *Poster presentation (Abstract number 3724).*

American Society of Hematology 49th Annual Meeting, Atlanta GA, USA, December 2007. *Poster presentation (Abstract number 1537).*

LIST OF PUBLICATIONS

Vilaine M, **Olcaydu D**, Harutyunyan A, Bergeman J, Tiab M, Ramée JF, Chen JM, Kralovics R, Hermouet S. Homologous recombination of wild-type JAK2, a novel early step in the development of myeloproliferative neoplasm. Blood. 2011 Dec 8;118(24):6468-70.

Klampfl T, Harutyunyan A, Berg T, Gisslinger B, Schalling M, Bagienski K, **Olcaydu D**, Passamonti F, Rumi E, Pietra D, Jäger R, Pieri L, Guglielmelli P, Iacobucci I, Martinelli G, Cazzola M, Vannucchi AM, Gisslinger H, Kralovics R. Genome integrity of myeloproliferative neoplasms in chronic phase and during disease progression. Blood. 2011 Jul 7;118(1):167-76.

Olcaydu D, Rumi E, Harutyunyan A, Passamonti F, Pietra D, Pascutto C, Berg T, Jäger R, Hammond E, Cazzola M, Kralovics R. The role of the JAK2 GGCC haplotype and the TET2 gene in familial myeloproliferative neoplasms. Haematologica. 2011 Mar;96(3):367-74.

Massera D, Mittermayer F, Gisslinger B, **Olcaydu D**, Wolzt M, Gisslinger H. Asymmetric dimethylarginine levels in essential thrombocythemia--a retrospective analysis. Ann Hematol. 2011 Sep;90(9):1105-6. Epub 2010 Dec 3.

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The 'GGCC' haplotype of JAK2 confers susceptibility to JAK2 exon 12 mutation-positive polycythemia vera. Leukemia. 2009 Oct;23(10):1924-6.

Olcaydu D, Harutyunyan A, Jäger R, Berg T, Gisslinger B, Pabinger I, Gisslinger H, Kralovics R. A common JAK2 haplotype confers susceptibility to myeloproliferative neoplasms. Nat Genet. 2009 Apr;41(4):450-4.