MEDICAL UNIVERSITY OF VIENNA

Identification of germline genetic factors contributing to susceptibility and clinical phenotype in myeloproliferative neoplasms

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Submitted by

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DECLARATION

The thesis is compiled in cumulative format. There are four manuscripts included in the thesis, the author of the current thesis being co-author on all manuscripts. The exact contributions of Ashot Harutyunyan and the short summary of the studies are described on the cover pages preceding the PDF versions of published manuscripts. Some experiments in section 3.2 have been performed by Christian Krendl and Tiina Berg. Figures 2, 22 and 23 are taken from other publications (also co-authored by Ashot Harutyunyan) and reprinted with the permission of the publisher. The rest of the thesis has been written by Ashot Harutyunyan.

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Manuscript # 2 was published in Nature Genetics: “A Common *JAK2* haplotype confers susceptibility to myeloproliferative neoplasms” by Olcaydu D., Harutyunyan A. et al., *Nat Genet* 2009; 41: 450-454. Reprinted with permission. Copyright Nature Publishing Group.

Manuscript # 3 was published in Haematologica: “The role of the *JAK2* GGCC haplotype and the *TET2* gene in familial myeloproliferative neoplasms” by Olcaydu D., Rumi E., Harutyunyan A. et al., *Haematologica* 2011; 96: 367-374. Reprinted with permission. Obtained from Haematologica/the Hematology Journal website, http://www.haematologica.org.

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TABLE OF CONTENTS

ABSTRACT

Myeloproliferative neoplasms (MPN) are a group of hematological malignancies primarily driven by somatic mutations and chromosomal aberrations. In the recent years significant amount of evidence has been accumulating on the importance of hereditary factors in MPN. Familial clustering, cases of biclonal MPN and phenotypic diversity in the presence of the same mutation have provided a solid basis for research in MPN germline genetics. Despite comprehensive clinical characterization of familial MPN, the major germline mutations responsible for MPN susceptibility have not been found. The same is true for the association of MPN with common SNPs in the population.

We performed a detailed study of germline genetic factors influencing MPN from multiple aspects. In order to study familial MPN, we first developed a nonparametric linkage analysis algorithm for high-throughput genotyping data. We validated the method on three families with known germline causative mutations and then applied it to a family with five affected MPN cases. Combining linkage analysis with exome sequencing and downstream validation of the identified hits, we found a mutation in *RBBP6* gene as the candidate susceptibility gene. Additional screening in other families with MPN yielded another two families with germline *RBBP6* mutations. RBBP6 is interacting with p53 and presumably the effect of mutation is mediated through p53 pathway. Moreover, we have shown an important role of p53 pathway in MPN, particularly in leukemic transformation, coming from the somatic genetics side.

We have also contributed to the understanding of the role of common germline predisposition to MPN. We have identified the strongest common predisposition for JAK2-positive MPN: the ‘GGCC’ haplotype spanning *JAK2* locus. Additionally, we have shown that although *JAK2* ‘GGCC’ haplotype confers the same risk in familial MPN, it cannot explain familial clustering of MPN.

Finally, we have identified a phenomenon of the interaction of somatic genetic aberrations and germline mutations. We have shown that a deleterious heterozygous mutation can influence disease phenotype when combined with acquired chromosomal aberration.

Overall, it seems that the inherited factors have quite an important role in MPN. The interconnection and overlap of somatic and germline genetics is particularly intriguing and will provide a new dimension in MPN research.

ZUSAMMENFASSUNG

Myeloproliferative Neoplasien (MPN) stellen eine Gruppe hämatologischer Erkrankungen dar, deren Ursache in erster Linie erworbene (somatische) Mutationen und chromosomale Veränderungen des Erbgutes sind. Forschungen der letzten Jahre haben zudem die Wichtigkeit von vererbten Faktoren in MPN gezeigt. Familiäre Häufungen, Fälle von biklonaler MPN sowie phänotypische Unterschiede bei Trägern der gleichen genetischen Veränderung rechtfertigen die Erforschung vererbter genetischer Veränderungen in MPN. Trotz umfangreicher Studien familiärer MPN wurden bisher keine Mutationen gefunden, die für MPN prädisponieren. Ebenso wurde bisher keine Assoziation von Einzelnukleotid-Polymorphismen (SNPs) mit MPN beschrieben.

Im Zuge dieser Doktorarbeit untersuchten wir vererbte genetische Faktoren, die einen Einfluss auf MPN haben, aus unterschiedlichen Perspektiven. Zu diesem Zweck, haben wir zunächst einen Algorithmus zur nichtparametrischen Kopplungsanalyse basierend auf Hochdurchsatz-Genotypisierungsdaten entwickelt. Wir validierten diese Methode an drei Familien mit verschiedenen Erkrankungen bei denen eine ursächliche, vererbte Mutation bekannt war. Danach untersuchten wir eine Familie mit fünf MPN Fällen, in welcher eine entsprechende Mutation noch nicht gefunden wurde. Die Kopplungsanalyse kombinierten wir mit einer Exom Sequenzierung. Nach Validierung der gefundenen genetischen Varianten konnten wir eine Mutation im *RBBP6* Gen nachweisen. In einer darauffolgenden Analyse von anderen Familien, in denen MPN gehäuft auftrat, identifizierten wir zwei weitere Familien mit vererbten Mutationen in *RBBP6*. Nachdem RBBP6 mit p53 interagiert, ist der Effekt der gefundenen Mutationen möglicherweise mit dem p53 Signaltransduktionsweg assoziiert. Wir konnten bereits vor dieser Studie durch eine Analyse somatischer Veränderungen zeigen, dass der p53 Signaltransduktionsweg eine wichtige Rolle in MPN spielt, im Besonderen bei der Transformation der Krankheit in eine akute myeloische Leukämie.

In einem zweiten Projekt konnten wir zeigen, dass ein häufig in der Bevölkerung vorkommender Haplotyp des *JAK2* Gens, der sogenannte ‘GGCC’ Haplotyp, die stärkste Prädisposition für JAK2-positive MPN darstellt. Das Risiko für Träger dieses Haplotyps eine MPN zu entwickeln ist zwar gleich für familiär gehäuft

auftretende und sporadische MPN, dennoch kann der Haplotyp die Häufung von MPN in Familien nicht erklären.

Zuletzt untersuchten wir das Zusammenwirken von vererbten und erworbenen genetischen Veränderungen in MPN. Eine heterozygote Mutation kann in Kombination mit einer erworbenen chromosomalen Veränderung die Charakteristika der Krankheit beeinflussen, wie wir an einem Beispiel zeigen konnten.

Zusammenfassend lässt sich sagen, dass erbliche genetische Faktoren eine wichtige Rolle in MPN spielen. Das Zusammenwirken von vererbten und erworbenen genetischen Faktoren ist besonders interessant und erweitert die MPN Forschung um eine neue Dimension.

1. INTRODUCTION

1.1 Overview of myeloproliferative neoplasms

Myeloproliferative neoplasms (MPN) are a heterogeneous group of malignant hematological disorders with the predominant involvement of myeloid lineage of hematopoiesis and characterized by hyperproliferation and accumulation of terminally differentiated blood cells. MPN comprise nine diagnostic units according to 2008 WHO classification (Tefferi & Vardiman, 2008). Polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) are considered as classical MPN by many experts in the field as a reminiscent of previous classification of myeloid disorders. The main subtypes are defined based on variable involvement of myeloid lineages in the disease process (Campbell & Green, 2006). Accordingly, the characteristic feature of polycythemia vera is increased red cell mass, hematocrit and hemoglobin values as a result of predominant involvement of erythroid lineage. Essential thrombocythemia presents with high platelet counts in peripheral blood as a consequence of megakaryocytic lineage hyperproliferation. Fibrosis of bone marrow is a key feature of primary myelofibrosis leading to extramedullary hematopoiesis. MPN has an inherent tendency of disease progression over time. Both PV and ET can progress to myelofibrosis (secondary myelofibrosis, sMF), while all three disease entities as well as sMF can transform into secondary acute myeloid leukemia (sAML) (Dingli et al, 2006). sAML has much worse prognosis than de novo AML, with the average lifespan of a few months (Ostgard et al, 2010). The risk of transformation to sAML depends on the disease subtype: it is low for PV and ET (1-3%), much higher in PMF (10-20%), and the highest in sMF (about 50%) (Passamonti et al, 2004). Besides the progression of the disease, the other main complications observed in patients are thrombosis and bleeding (Arellano-Rodrigo et al, 2006; Cervantes et al, 2006). In some cases a thrombotic or hemorrhagic event can be the first presentation of MPN.

The annual incidence of MPN is about 1-2 per 100,000 and as the patients usually survive for many years, the prevalence of the disease in the population is about 10-fold higher (Johansson et al, 2004). The age of onset is usually after 50 years, although there are many reported younger patients, especially in familial cases of the disease (Bellanne-Chantelot et al, 2006). The incidence of PV and ET is

similar, while PMF is rarer by 4-5 fold. The average lifespan for PV and ET is more than ten years; often patients die of unrelated causes as MPN are mainly diseases of the elderly. The main disease-related causes of death of the patients are thrombotic and hemorrhagic complications, as well as disease evolution (Campbell & Green, 2006).

The clinical presentation of the patients is based on underlying pathological processes. The symptoms are consequences of elevated red cell mass, platelet and white blood cells levels. Often patients are asymptomatic for a long time, and sometimes the initial presentation is a major complication of thrombosis or bleeding. Some patients experience itching after warm bath, fatigue, headaches. A specific but rare symptom of PV and ET is erythromelalgia. A proportion of patients have splenomegaly, especially in cases of myelofibrosis. Myelofibrosis patients, due to anemia and neutropenia, also have higher susceptibility to infections and shortness of breath (Campbell & Green, 2006).

The treatment of MPN patients is aimed to relieve constitutional symptoms and to prevent complications. Many patients survive for years with little or no treatment. In a sizeable group of patients, bloodletting, the historical treatment option, could be enough alone to control the symptoms. Most of the patients receive low-dose aspirin (Santos & Verstovsek, 2012) or other antiplatelet agents, such as anagrelide (Gisslinger et al, 2013) to prevent thrombotic complications. As eventually many patients develop bone marrow fibrosis, hydroxyurea is being administered to sizeable number of patients to prevent it and keep cell numbers in control (Barbui et al, 2011; Cortelazzo et al, 1995). Historically patients have been receiving more aggressive treatment, particularly with alkylating agents such as pipobroman, chlorambucil, busulfan, etc., but since these drugs frequently cause secondary leukemias, they have been mostly taken out from clinical use (Barbui, 2004). The leukemogenicity of widely used hydroxyurea has been debated, with contradictory results from different studies (Gisslinger et al, 2013; Kiladjian et al, 2006b). The patients with myelofibrosis who develop severe anemia, also receive frequent blood transfusions (Campbell & Green, 2006). In case of leukemic transformation, when the clinical picture is that of AML, patients receive aggressive chemotherapy, although these treatments usually fail to induce clinical remission (Ostgard et al, 2010). The only curative treatments so far in MPN have been reported to be interferon-alpha, which achieves complete cure in about 20% of patients but is not well tolerated by

patients (Kiladjian et al, 2008; Kiladjian et al, 2006a; Kiladjian et al, 2011) and allogenic bone marrow transplant (McLornan et al, 2012). Recently a new class of drugs, JAK2 inhibitors, was developed, based on the fact that *JAK2* gene is frequently mutated in MPN (Harrison et al, 2012; Tefferi et al, 2011; Verstovsek et al, 2012b). So far clinical trials have shown only limited success of these drugs: while they improve the constitutional symptoms, there is not much change in the *JAK2* mutational burden (Vaddi et al, 2012; Verstovsek et al, 2012a).

The other diseases included in MPN group are BCR-ABL1-positive chronic myelogenous leukemia, chronic neutrophilic leukemia, hypereosinophilic syndrome, mast cell disease, chronic eosinophilic leukemia not otherwise specified, and unclassifiable MPN (Tefferi & Vardiman, 2008). Although these diseases have some shared pathogenesis and clinical picture (hyperproliferation of one or more myeloid lineages, some mutations and chromosomal aberrations present in different types) there are also a number of differences, particularly in the genes mutated and prevalence of mutations, in the myeloid lineage involved and in the disease course.

There is a group of related myeloid disorders that are classified as MDS/MPN. These diseases have overlapping clinical and pathological features of MPN and myelodysplastic syndromes (MDS). There are four disease entities in this category: chronic myelomonocytic leukemia (CMML), juvenile myelomonocytic leukemia (JMML), atypical chronic myeloid leukemia (aCML) and MDS/MPN unclassifiable (Vardiman et al, 2009). Another group includes myeloid neoplasms associated with eosinophilia and abnormalities of PDGFRA, PDGFRB or FGFR1. Finally, two big groups of myeloid malignancies are acute myeloid leukemias (AML) and myelodysplastic syndromes. AML is characterized by rapid onset and progression of the disease, presence of undifferentiated blast cells in the peripheral blood and block in myeloid differentiation. MDS are manifested with various cytopenias and dysplasia in one or more myeloid lineages in combination with bone marrow hypercellularity. The current classification of myeloid neoplasms is mainly focused on clinicopathological features of the disease and morphology of blood cells and bone marrow, but it also takes into account various mutations and chromosomal aberrations (Vardiman, 2010; Vardiman et al, 2009). With more and more new genetic lesions described in myeloid malignancies, the classification will soon rely heavier on molecular features of the disease.

1.2 Genetics of myeloproliferative neoplasms

MPN is a form of chronic leukemia, and thus the somatic mutations and chromosomal aberrations are the main drivers of the disease. However, the germline factors have also been shown to influence the susceptibility to the disease, as well as some features of pathogenesis. Over the years many recurrent genetic lesions have been discovered in MPN which shed light on the pathogenesis of the disease (Milosevic & Kralovics, 2013). Initially chromosomal aberrations were detected using classical karyotyping (Gangat et al, 2008; Gangat et al, 2009; Hussein et al, 2009b; Panani, 2007), but in last few years widespread use of array competitive genomic hybridization (CGH) technology (or SNP arrays) enabled researchers to find smaller scale lesions previously undetectable by classical cytogenetic analyses (Klampfl et al, 2011; Rumi et al, 2011; Stegelmann et al, 2010; Thoennissen et al, 2010). Similarly the advances in sequencing technologies, particularly second generation sequencing, facilitated the discovery of point mutations in various genes (Ernst et al, 2010; Ley et al, 2010; Maxson et al, 2013; Piazza et al, 2013). While much progress has been made in somatic genetics of MPN, there is still a lot to be done in germline genetics. Both germline and somatic genetic factors important in MPN pathogenesis are discussed in the following sections.

1.2.1 Somatic point mutations and chromosomal aberrations in MPN pathogenesis

Both point mutations and chromosomal aberrations that are acquired somatically play defining role in MPN pathogenesis. In many cases these aberrations are acting in combination, complementing each other. A somatic point mutation may achieve full homozygosity by acquired uniparental disomy (UPD), or it may become hemizygous by the deletion of the second copy of the gene. Finally, amplification of a part of a chromosome may amplify the gene copy with the mutation, thus increasing allelic burden (Kralovics, 2008; Milosevic & Kralovics, 2013). For that reason, chromosomal aberrations and point mutations that are affecting the same gene are discussed together below. The frequencies of point mutations in genes most frequently affected in MPN are shown on **Figure 1**, while the overview of the recurrent chromosomal aberrations is shown on **Figure 2**.

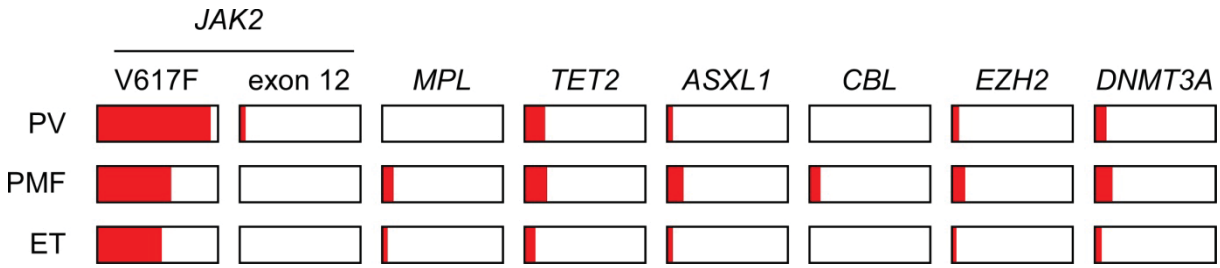


Figure 1. The distribution of common somatic mutations in the three MPN subtypes. Red bars indicate the approximate frequency of mutations in each diagnostic category.

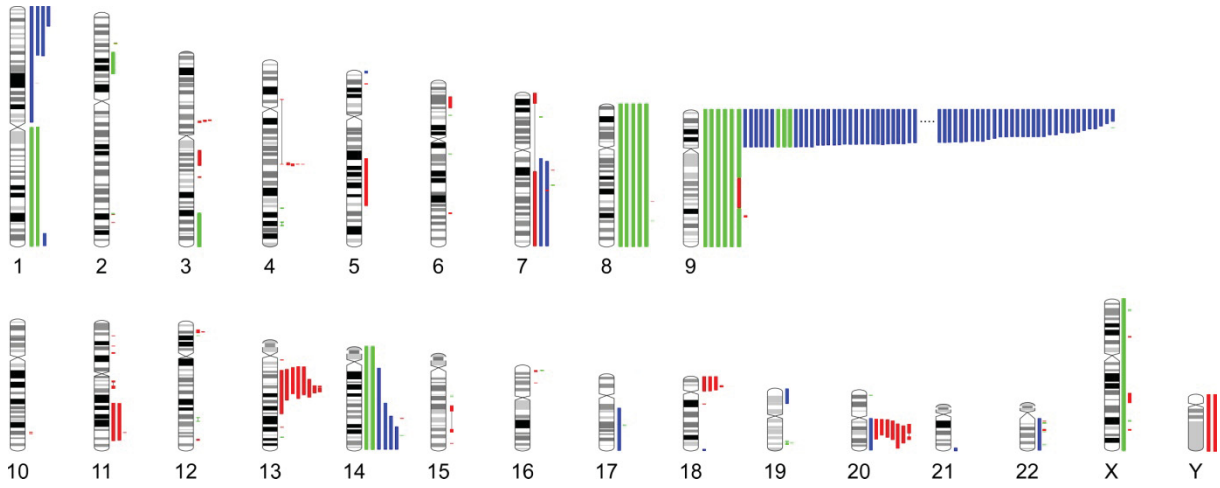


Figure 2. The distribution of chromosomal aberrations detected by Affymetrix SNP 6.0 arrays in chronic phase MPN patients. Bars correspond to the size and the position of aberrations; green indicates gains; red, deletions; and blue, UPDs. (*Figure reprinted from Klampfl T., Harutyunyan A. et al., originally published in Blood. Klampfl T., Harutyunyan A. et al. Genome integrity of myeloproliferative neoplasms in chronic phase and during disease progression. Blood 2011;118(1):167-176. © the American Society of Hematology*).

By far the most common chromosomal aberrations in MPN (in more than 30% of patients) are acquired uniparental disomies of short arm of chromosome 9 (9p UPD) (Klampfl et al, 2011; Kralovics et al, 2002). Additionally, amplifications of chromosome 9p and trisomy 9 are also recurrently found in MPN (Klampfl et al, 2011). Following up on the identification of 9p UPDs in MPN, in 2005 four groups reported on the identification of a point mutation V617F in the Janus kinase 2 gene (*JAK2*) as frequent in MPN and associated with large scale chromosome 9 lesions (Baxter et al, 2005; James et al, 2005; Kralovics et al, 2005; Levine et al, 2005). Eventually JAK2-V617F became the main hallmark of MPN since more than half of

the patients carry that mutation (Jones et al, 2005). V617F mutation is particularly frequent in PV, reaching 95% of the cases and is also frequently associated with 9p UPD (Campbell & Green, 2006). In contrast, 9p UPD is rare in ET patients who are JAK2-V617F-positive (Klampfl et al, 2011; Stegelmann et al, 2010). Subsequently, different mutations of *JAK2* were found in exon 12 of the gene in a subgroup of PV patients who are JAK2-V617F-negative (Pietra et al, 2008; Scott et al, 2007). Taken together, *JAK2* mutations are present in almost all cases of PV. The amplifications of chromosome 9p and trisomy 9 are also associated with JAK2-V617F mutation (Klampfl et al, 2011). JAK2 is a receptor-associated tyrosine kinase which is coupled with many cytokine receptors, including erythropoietin receptor (EPOR) and thrombopoietin receptor (MPL). Upon activation following cytokine binding to receptors, JAK2 phosphorylates signal transducers and activators of transcription (STATs) which induce the expression cell proliferation genes (Funakoshi-Tago et al, 2010; Lu et al, 2005). V617F and exon 12 mutations make *JAK2* constitutively active and thus cells are proliferating faster even in the absence of cytokines. These mutations are located in the pseudokinase domain of JAK2 and remove its autoinhibitory effect, thus keeping JAK2 constantly active (Kralovics et al, 2005). *JAK2* mutations are sometimes called 'phenotypic' in the sense that the acquisition and clonal predominance of these mutations usually coincides with the initial clinical presentation of the disease (Kralovics, 2008).

Not long after the discovery of *JAK2* mutations, recurrent mutations in thrombopoietin receptor gene, *MPL*, were found in a minority of cases of JAK2-negative ET and PMF (Pikman et al, 2006; Staerk et al, 2006). The somatic *MPL* mutations are affecting the residue W515 (W515L, W515R, W515A, W515K, W515S), making it cytokine independent (Pardanani et al, 2006b; Pikman et al, 2006; Staerk et al, 2006). *MPL* mutations are sometimes coupled with UPD of chromosome 1p which makes the *MPL* mutation fully homozygous (Buxhofer-Ausch et al, 2009; Szpurka et al, 2009). Although *MPL* mutations are present only in about 1-5% of ET and PMF cases, they are also considered 'phenotypic' mutations as *JAK2* (Kralovics, 2008).

The defects of the long arm of chromosome 4, namely 4q UPDs and deletions, are found in a fraction of MPN patients (Delhommeau et al, 2009; Klampfl et al, 2011). The use of SNP arrays allowed the identification of small focal deletions on 4q, which eventually pointed to the candidate gene, ten-eleven translocation oncogene

family member 2 (*TET2*) (Delhommeau et al, 2009; Langemeijer et al, 2009). *TET2* point mutations were found to be strongly associated with chromosome 4q defects (Delhommeau et al, 2009; Langemeijer et al, 2009; Tefferi et al, 2009). Large scale screenings of MPN cohorts for *TET2* mutations determined that it is one of the most common mutations in MPN, reaching up to 10-15% in PV and PMF, being less frequent in ET (Tefferi et al, 2009). TET2 was shown to be the enzyme that converts 5-methylcytosine to 5-hydroxymethylcytosine, which in a subsequent process is converted back to unmethylated cytosine (Ito et al, 2011; Ko et al, 2010). In effect, TET2 is involved in DNA demethylation. *TET2* was the first gene among epigenetic regulators discovered to be mutated in MPN underlining the importance of epigenetic regulation pathways in MPN. In contrast to *JAK2* and *MPL* which are much less frequently mutated in other myeloid malignancies, TET2 mutations are very common in MDS, MDS/MPN, AML, in some specific disease entities reaching 50% (Delhommeau et al, 2009; Langemeijer et al, 2009).

Chromosome 11q abnormalities are another set of recurrent aberrations in MPN (Dunbar et al, 2008; Grand et al, 2009; Klampfl et al, 2011). The main target of those aberrations, particularly UPDs, has been found to be Casitas B-lineage lymphoma proto-oncogene (*CBL*) which is an E3 ubiquitin ligase (Dunbar et al, 2008; Grand et al, 2009; Sanada et al, 2009). CBL functions as a regulator of cytokine signaling since it ubiquitinates tyrosine kinases (e.g. JAK2), cytokine receptors (EPOR, MPL) and other molecules involved in signaling pathways (Saur et al, 2010; Schmidt & Dikic, 2005). The mutations in *CBL* are thought to be loss-of-function, are mainly affecting exons 8 and 9, which code for RING finger domain (Grand et al, 2009). Thus, mutant protein does not have E3 ligase activity. However, some reports have also suggested that *CBL* mutations are gain-of-function or dominant-negative (Sanada et al, 2009). *CBL* mutations are frequently found in PMF (up to 10%), while the cases of ET and PV with *CBL* mutations are very rare (Grand et al, 2009). *CBL* mutations are also prevalent in a wide range of myeloid malignancies, more frequently in MDS, MDS/MPN and sAML (Sanada et al, 2009).

Another set of recurrent chromosomal aberrations in MPN on chromosome 7, seem to have a complex pattern of targets. Chromosome 7 deletions and monosomies are very common in AML, while 7q UPDs are frequently found in MDS and rarely also in MPN (Klampfl et al, 2011). EZH2 (enhancer of zeste homolog 2), a histone methyltransferase and the catalytic subunit of polycomb repressive complex

2 (PRC2) was shown to be the main target of 7q UPDs (Ernst et al, 2010; Nikoloski et al, 2010). *EZH2* mutations are more common in PMF (about 10%), and 1-5% in ET and PV, mostly associated with 7q UPDs (Guglielmelli et al, 2011). This is the second gene mutated in MPN and other myeloid neoplasms that is involved in epigenetic regulation. Interestingly, no *EZH2* mutations were found in AML cases with monosomy 7 or deletion 7q, suggesting that there might be other targets on chromosome 7 (Ernst et al, 2010). Using SNP arrays, we have mapped one of the targets to single gene, *CUX1* (cut-like homeobox 1) (Klampfl et al, 2011). CUX1 is a transcription factor and regulates various cellular processes. It was later functionally shown that CUX1 haploinsufficiency gives proliferative advantage to hematopoietic progenitor cells and enhances the engraftment efficiency in a transplant model (McNerney et al, 2013). Moreover, previously we have also mapped the target of 7p deletions to a single gene, *IKZF1* (Ikaros), another transcription factor (Jager et al, 2010). *IKZF1* deletions are found in more than 80% of BCR-ABL1-positive acute lymphoblastic leukemia (ALL) (Mullighan et al, 2008). There is not much evidence of point mutations in *CUX1* and *IKZF1* so far, but multiple recurrent focal deletions in those regions are proving their important role in MPN as well as other myeloid malignancies. Overall there are at least 3 targets on chromosome 7 (*EZH2*, *CUX1*, *IKZF1*) and there might be even more yet to be discovered. This fact highlights the complexity of chromosomal aberrations and suggests that some of the aberrations might have multiple targets.

Mutations in additional sex combs like 1 (*ASXL1*) are rather frequently found in MPN and other myeloid disorders (Carbuccia et al, 2009). ASXL1, being a member of Polycomb group of proteins, functions as a regulator of transcription (Cho et al, 2006). *ASXL1* mutations are found in about 10-15% of PMF and sMF cases, while they are much less prevalent in PV and ET (Carbuccia et al, 2009). The mutations in *ASXL1* are also not MPN-specific, being present in MDS and MDS/MPN (Gelsi-Boyer et al, 2009). Although *ASXL1* is located on chromosome 20, a region of frequent chromosomal aberrations in MPN, these mutations have not been linked with deletions of 20q.

Mutations in DNA cytosine methyltransferase 3 alpha (*DNMT3A*) were discovered to be common in AML (Ley et al, 2010). Subsequent studies have shown that *DNMT3A* mutations are present in other myeloid malignancies (Walter et al, 2011), including MPN. About 3% of ET, 7% of PV and up to 15% of PMF patients

carry *DNMT3A* mutations (Abdel-Wahab et al, 2011; Stegelmann et al, 2011), which makes it one of the commonly mutated genes in MPN. DNMT3A is a *de novo* DNA methyltransferase and the presence of mutations in this gene underline the important role of epigenetic regulation in MPN as well as other myeloid malignancies.

Due to the widespread use of second generation sequencing and other modern genetic technologies, more and more genes are found to be mutated in a fraction of MPN patients. Some of the recently found and notable ones are splicing machinery mutations (*SF3B1*, *SF3A1*, *SRSF2*, *SF1*, *U2AF1*, *ZRSR2*, etc.) (Papaemmanuil et al, 2011; Yoshida et al, 2011), *LNK* or *SH2B3* adapter protein mutations (Pardanani et al, 2010a), *NFE2* transcription factor mutations (Jutzi et al, 2013). Some other mutations have been found in subtypes of MPN by its WHO definition or MDS/MPN but are absent in “classical” MPN, such as *CSF3R* mutations in chronic neutrophilic leukemia and atypical CML (Maxson et al, 2013; Pardanani et al, 2013) or *SETBP1* mutations in atypical CML (Piazza et al, 2013).

Deletions of 20q are present in a fraction of MPN patients and constitute one of the most frequent chromosomal aberrations in the chronic phase of the disease (Klampfl et al, 2011). Numerous studies have addressed the role of 20q deletions in MPN after they were discovered by classical karyotyping, but none of them succeeded to identify the target (Bench et al, 2000; Gondek et al, 2008; Schaub et al, 2009). Recently it was reported that 20q deletions are targeting an imprinted gene cluster and simultaneous deletion of paternally expressed copies of *SGK2* and *L3MBTL1* results in complete loss-of-function of these genes (Aziz et al, 2013). In a very interconnected and sophisticated manner, combined inhibition of the two genes enhances the erythroid lineage and also significantly increases MYC levels (Aziz et al, 2013). This is an unusual example of the mechanism of action of chromosomal aberrations and once again demonstrates the complexity of MPN genetics. Whether the other chromosomal aberrations act through similar mechanisms is yet to be determined.

Alongside 20q deletions, deletions located in the long arm of chromosome 13 are among the most frequent in chronic MPN (Klampfl et al, 2011). The target of these deletions has not been identified yet, but the mapping of smallest commonly deleted region (CDR) resulted in a region containing several genes including retinoblastoma 1 (*RB1*) (Klampfl et al, 2011). *RB1* is a very well known tumor suppressor and is deleted or mutated in many different cancers. Although *RB1* has

been frequently suggested to be the 13q target, its role in MPN is not clear yet. So the target of chromosome 13q deletions so far remains elusive and a matter of speculations.

Trisomy 8 is recurrently found in MPN, as well as MDS (Klampfl et al, 2011; Stegelmann et al, 2010). The target is not found, although since *MYC* is located on chromosome 8, it has been speculated to be the target.

To complete the list of the most common chromosomal aberrations in chronic phase MPN, it is important to mention chromosome 14q UPDs. These aberrations are rather common, possibly being the second most common UPD in MPN after 9pUPD and in the same frequency range as 1p UPDs (Klampfl et al, 2011). 14q UPDs are commonly observed in chronic phase and advanced phase (sMF) MPN, but are not enriched in sAML (Klampfl et al, 2011). Although this aberration is rather frequent, the target has not been identified to date. 14q UPD is also common in MDS (Gondek et al, 2008) and is one of the most common aberrations found in healthy subjects, along with chromosome 13q and 20q deletions (Jacobs et al, 2012; Laurie et al, 2012). Most likely this reflects the patients in pre-clinical phase of MPN.

There are some chromosomal aberrations that are rare in chronic phase MPN but are enriched with disease progression (sMF and sAML), most notably amplifications of chromosome 1q and deletions of 5q (Klampfl et al, 2011; Thoennissen et al, 2010). These aberrations are also found in other myeloid malignancies such as MDS and AML. Particularly 5q deletions are rather frequent in AML and MDS and are even used for disease classification (Gondek et al, 2008; Milosevic et al, 2012).

In the same context, there are a number of genes mutated in advanced phase MPN (sMF and sAML), but these mutations are almost absent in chronic phase. The examples include *IDH1* and *IDH2* (Green & Beer, 2010; Pardanani et al, 2010c; Tefferi et al, 2010a), *RUNX1* (Beer et al, 2010a; Ding et al, 2009), *NPM1* (Falini et al, 2005), *FLT3* (Milosevic et al, 2012), etc.. These mutations are quite common in MDS and AML and most likely are involved in leukemic transformation of MPN (Milosevic et al, 2012).

1.2.2 Hereditary factors in myeloproliferative neoplasms

The myriad of somatic point mutations and chromosomal aberrations creates

an impression that only somatic genetic alterations are important in MPN pathogenesis. This statement is partially true, since indeed somatic lesions are the main driving forces of the disease. However, there are a number of important aspects of MPN that cannot be explained solely by somatic mutations and point towards certain role of germline genetic factors. Germline factors can predispose to MPN, to acquisition of specific point mutations or chromosomal aberrations, as well as can influence the emergence of certain clinical symptoms, the clinical course or the complications of the disease (Harutyunyan & Kralovics, 2012).

Some MPN patients are shown to be “biclonal”, i.e. there are at least two independent sources of malignant cells in the blood which cannot be traced back to a common ancestral cell (demonstrated by different X chromosome inactivation pattern of the two malignant clones) (Beer et al, 2009; Beer et al, 2010b; Hussein et al, 2009a; Kralovics, 2008). Since the probability of two independent clonal outgrowths in a single patient is too low to be encountered, the only reasonable explanation is the existence of inherent genetic predisposition in such individuals (Harutyunyan & Kralovics, 2012).

Phenotypic diversity of MPN is another aspect of the disease that so far cannot be fully explained by somatic mutations. Specifically, the development of three different subtypes of the disease (PV, ET and PMF) in different patients who carry the same JAK2-V617F mutation so far has not been sufficiently clarified. Although it is plausible that some as yet unidentified somatic mutations are defining the disease phenotype, the influence of germline variants is also a valid alternative. Interestingly, mouse models of MPN are also giving some hints that germline background might be important for this disease. JAK2-V617F transgenic mouse models have PV-like or ET-like phenotype, depending on mutation dosage (Tiedt et al, 2008). However, the phenotype, e.g. the degree of bone marrow fibrosis also depends on the mouse strain used (Balb/c mice have more fibrosis compared to C57Bl/6) (Wernig et al, 2006; Zaleskas et al, 2006).

There is also rather wide variability in clinical course of the disease, namely the rate of complications. Some patients have significantly higher risk than others of developing thrombotic complications, splenomegaly, myelofibrosis, etc., even while presenting with similar molecular defects (Casini et al, 2013). This variability has not been explained by the known susceptibility factors for those complications (e.g. thrombosis risk factors), therefore MPN-specific, non-somatic susceptibility factors

are supposed to exist.

It has also been shown that relatives of MPN patients have 6-8 fold higher risk of developing MPN (Landgren et al, 2008). This goes in line with the existence of so-called “MPN families”, when several members in a family are affected by the disease (Kralovics et al, 2003). Overall, it has been shown that about 5-10% of MPN cases are familial (Rumi, 2008; Rumi et al, 2007). Familial clustering of MPN is one of the major arguments supporting the contribution of germline susceptibility to the disease. There have been a number of studies on familial MPN, mostly providing clinical and molecular characteristics of the patients and also checking for germline mutations in some candidate genes, although with not much success regarding the latter aspect (Bellanne-Chantelot et al, 2006; Kralovics et al, 2003). However, these studies provided the proper characterization of “true” familial MPN and their differentiation from MPN-like disorders. Additionally, some reports have studied the distribution of known MPN mutations (e.g. *JAK2*, *MPL*) in familial MPN cases and found that it is not significantly different from sporadic MPN and that those mutations have somatic origin (Bellanne-Chantelot et al, 2006; Rumi et al, 2006). This means that those mutations cannot explain familial clustering of MPN, as well as that familial and sporadic MPN have the same somatic mutational drivers of the disease and thus similar disease pathogenesis.

Familial MPN are similar to sporadic MPN and display low penetrance, clonal hematopoiesis, involvement of multiple hematopoietic lineages, existence of somatic mutations in many patients and progression of the disease (sMF, sAML) (Rumi, 2008; Rumi et al, 2007; Rumi et al, 2006). These features differentiate familial MPN from MPN-like disorders which will be discussed in more detail in the next section.

There is a notable clinical and genetic heterogeneity in MPN families. First of all, it concerns MPN diagnosis of family members. While in some families the affected members have the same MPN subtype (PV, ET, or PMF), in other families patients with different MPN subtypes are present in the same pedigree (Bellanne-Chantelot et al, 2006; Kralovics et al, 2003; Rumi et al, 2007). The same is true regarding somatic mutations, i.e. in some families all affected members have JAK2-V617F mutations, in others there are different mutations in different patients (JAK2-V617F, JAK2-exon12, MPL), in yet other families some members have somatic mutations in known genes, the others are negative, and finally some MPN families are described by the absence of any known MPN mutations (Bellanne-Chantelot et

al, 2006; Pardanani et al, 2006a; Rumi et al, 2006). Significant variation exists also regarding the complications or progression of the disease, with some families showing unusually increased tendency to certain complications (Bellanne-Chantelot et al, 2006; Rumi, 2008). There is also variability in the penetrance of the disease ranging from low penetrance to almost complete penetrance, and in the inheritance mechanism, resembling autosomal dominant or recessive (Landgren et al, 2008; Rumi et al, 2007). However, overall familial MPN seem to have autosomal dominant inheritance pattern with low penetrance (Harutyunyan & Kralovics, 2012; Rumi, 2008).

To conclude regarding familial MPN, it is important to note that despite comprehensive characterization and studies of large cohorts of families, the germline mutations responsible for those cases have not been identified so far.

There are also interesting developments in the area of common predisposition in MPN. Using candidate gene approach, one study found several SNPs in the region of *JAK2* gene to be associated with PV phenotype when compared to ET (Pardanani et al, 2008). This can have important implications for the germline genetics field of MPN, since this is the first report showing that a germline SNP can influence the susceptibility to a specific subtype of the disease. Subsequent studies will show whether there are more common SNPs involved in MPN predisposition or this one was just an exception.

1.2.3 Genetics of MPN-like disorders

A group of inherited hematological disorders present with MPN-like symptoms and in many cases it is rather difficult to differentiate them. These MPN-like disorders have more benign clinical course and some important differences from MPN. In contrast to MPN, they have almost complete penetrance, polyclonal hematopoiesis, involve single hematopoietic lineage (erythroid or megakaryocytic), do not carry somatic mutations and usually there is no progression of the disease (Percy & Rumi, 2009; Rumi, 2008). Although there are some reports that these patients develop thrombotic and hemorrhagic complications similar to MPN, usually the disease course is benign (Ding et al, 2004; Teofili et al, 2007; Teofili et al, 2010). MPN-like disorders are subdivided into hereditary erythrocytosis and hereditary thrombocytosis, and each of these can be primary or secondary (Percy & Rumi,

2009). The genetic basis of these disorders has been partially elucidated and will be discussed in more detail. The underlying germline genetic defect in MPN-like disorders is driving the disease itself, without the need for further somatic mutations.

Hereditary erythrocytosis. The patients are characterized by elevated red blood cell levels in the absence of changes in platelet or white blood cell levels (Percy & Rumi, 2009). In case of primary familial and congenital polycythemia, many patients are found to carry truncating mutations in *EPOR* gene, which result in the removal of its inhibitory domain (de la Chapelle et al, 1993). The transmission of the disease is autosomal dominant, the serum levels of erythropoietin are very low and the erythrocytosis is primary. In contrast, other patients carrying mutations in oxygen-sensing pathway genes (*VHL*, *EGLN1*, *EPAS1*) or genes affecting oxygen affinity of hemoglobin (*HBB*, *BPGM*) have either high of inappropriately normal levels of erythropoietin and the cause of high red blood cell count is secondary to high erythropoietin levels (Albiero et al, 2011; Ang et al, 2002a; Ang et al, 2002b; Percy et al, 2008; Rosa et al, 1978; Wajcman & Galacteros, 2005). The patients have increased risk for thrombosis but do not show signs of disease progression. Except for *VHL* mutations, when the inheritance is autosomal recessive, other cases of erythrocytosis are transmitted in autosomal dominant manner (Percy & Rumi, 2009).

Hereditary thrombocytosis. As in case of hereditary erythrocytosis, hereditary thrombocytosis can also be primary and secondary. Mutations in thrombopoietin (*THPO*) gene result in more stable mRNA and thus increased amounts of thrombopoietin, which in turn causes secondary thrombocytosis (Liu et al, 2008; Wiestner et al, 1998).

A proportion of hereditary primary thrombocytosis is caused by S505N mutation in *MPL* gene (Ding et al, 2004; El-Harith el et al, 2009; Moliterno et al, 2004; Teofili et al, 2007). This mutation is different from somatic mutations in MPL that are found in MPN cases. The effect of *MPL* mutations is constitutive hyperactivity of the receptor and consequently JAK-STAT pathway. In these patients the levels of thrombopoietin are very low (Percy & Rumi, 2009; Rumi, 2008). MPL-S505N mutation has been found in a few familial cases of pediatric ET (which actually are not ET, but rather thrombocytosis patients) and has been shown to be identical-by-descent (Teofili et al, 2007; Teofili et al, 2010).

Recently germline mutations in *JAK2* (V617I, R564Q, H608N) were shown to cause hereditary primary thrombocytosis with similar features as germline *MPL*

mutations (Etheridge et al, 2011; Mead et al, 2012; Rumi et al, 2012b). The levels of thrombopoietin are also low and the mutations result in hyperactivity of JAK-STAT pathway, demonstrated by hyperphosphorylation of STATs (Mead et al, 2013). These mutations seem to be milder than V617F, the latter is embryonically lethal and can be acquired only somatically.

Table 1. Summary of identified germline mutations predisposing to MPN-like disorders.

Gene	Germline mutation	Disease	Inheritance	Clonality	Lineage involvement
JAK2	missense mutations (V617I, H564Q, H608N)	hereditary thrombocytosis / ET	autosomal dominant	polyclonal	megakaryocytic
MPL	missense mutation (S505N)	hereditary thrombocytosis	autosomal dominant	polyclonal	megakaryocytic
THPO	splice site/ missense/ regulatory mutations	hereditary thrombocytosis	autosomal dominant	polyclonal	megakaryocytic
EPOR	truncating mutations	primary familial and congenital erythrocytosis	autosomal dominant	polyclonal	erythroid
VHL	loss-of function mutations	hereditary secondary erythrocytosis	autosomal recessive	polyclonal	erythroid
EPAS1	missense mutations	hereditary secondary erythrocytosis	autosomal dominant	polyclonal	erythroid
EGLN1	loss-of function mutations	hereditary secondary erythrocytosis	autosomal dominant	polyclonal	erythroid
HBB	high oxygen affinity variants	hereditary secondary erythrocytosis	autosomal dominant	polyclonal	erythroid
BPGM	loss-of function mutations	hereditary secondary erythrocytosis	autosomal dominant	polyclonal	erythroid

The mutations so far described in hereditary erythrocytosis (listed in the **Table 1**) and thrombocytosis explain only a certain proportion of the families. Only about 15% of primary erythrocytosis is explained by *EPOR* mutations, while *MPL*, *JAK2*

and *THPO* explain about 20-25% of hereditary thrombocytosis (Percy & Rumi, 2009; Rumi, 2008). This implies that there are more genes yet to be discovered to be causative in the remaining majority of the cases. The high penetrance of the disease facilitates the identification of key germline mutations in these families in contrast to true MPN where the low penetrance is a major problem.

1.2.4 Hereditary factors in other myeloid malignancies

Germline genetic factors are also important in other myeloid cancers such as MDS and AML. A number of highly penetrant mutations have been described to confer susceptibility to these diseases, particularly in the familial predisposition context. In this sense the germline genetics field is more advanced for MDS/AML compared to MPN.

The familial cases of MDS and AML can be divided into two main groups: syndromic and non-syndromic (or pure). Syndromic cases are caused by mutations in various genes which also result in various different non-hematological symptoms. In essence, MDS and/or AML are just one of the features of the syndrome (Liew & Owen, 2011). On the other hand, in non-syndromic cases hematological phenotype is the only manifestation of the disease. Some examples of those will be discussed below. In most cases, the inheritance is autosomal dominant.

Germline mutations in *RUNX1*, a member of core binding factor transcription complex, are causing familial platelet disorder with propensity to myeloid malignancy (Song et al, 1999). Many such families have been described so far and the phenotypic features are quite variable, ranging from mild platelet abnormalities and thrombocytopenia up to full-blown MDS and AML. Additionally, some cases with T-cell acute lymphoblastic leukemia (ALL) have been described (Liew & Owen, 2011). The development of MDS and AML has quite a long period of latency in these familial cases since additional somatic lesions are required to drive the leukemia. In many cases the second hit is the loss of the second *RUNX1* allele, by point mutation of a deletion (Liew & Owen, 2011). Another class of familial MDS/AML are caused by small deletions on chromosome 21 spanning *RUNX1* locus, with essentially the same pathogenesis, although sometimes these are associated with non-hematological symptoms as well (Huret et al, 1995). As already mentioned in the section above, *RUNX1* somatic mutations can also be acquired somatically in myeloid malignancies,

particularly in sAML (Ding et al, 2009).

CEBPA germline mutations have been implicated in familial cases of AML (Smith et al, 2004). The development of AML has a long period of latency, consistent with the acquisition of additional mutational hits. These patients have also been reported to present with eosinophilia (Carmichael et al, 2010).

Mutations in *GATA2* in germline context have been recently described as a cause of familial MDS in four families (Hahn et al, 2011). In contrast to *RUNX1* or *CEBPA* mutations, the patients do not have additional hematological symptoms and only have typical MDS features. As for the other cases of familial MDS, the disease requires and is mainly driven by additional somatically acquired mutations and chromosomal aberrations (Hahn et al, 2011).

An autosomal dominant form of familial MDS/AML is associated with germline mutations in telomere components *TERC* (telomerase RNA component) or *TERT* (telomerase reverse transcriptase) (Kirwan et al, 2009; Yamaguchi et al, 2003). Telomerase is expressed in stem cells and is necessary for highly proliferative tissues such as bone marrow to maintain the proper length of telomeres and hence the integrity of chromosomes. Loss-of-function mutations in telomerase components result in telomere shortening and eventually in acquisition of additional chromosomal aberrations. The latter are, in turn, manifesting by the development of MDS/AML. An important feature of this form of familial MDS/AML is the disease anticipation when each following generation has earlier age of disease onset. This is explained by the gradual germline shortening of telomeres in each generation (Liew & Owen, 2011).

An interesting class of the familial MDS is the familial monosomy 7. The members of the family affected by MDS/AML carry monosomy of chromosome 7 (or in some cases partial loss of chromosome 7) which has somatic origin (Gaitonde et al, 2010). This loss of chromosome is not consistent with Knudson's two-hit hypothesis since both paternal and maternal chromosomes can be lost in the different members of the same family essentially ruling out that the causative gene is on chromosome 7 (Minelli et al, 2001). Since chromosome 7 abnormalities are common in myeloid malignancies, it is not surprising and likely has the same value as monosomy 7 in sporadic cases of MDS and AML. The predisposition gene is so far unknown, as well as the mechanism by which monosomy 7 is consistently somatically acquired (Liew & Owen, 2011).

Although mutations in a number of genes responsible for familial MDS/AML

have been identified, those explain only a proportion of familial occurrence of the disease. For the rest of the pedigrees, the cause remains so far unknown.

Germline mutations in *CBL* are responsible for familial cases of juvenile myelomonocytic leukemia (JMML) (Niemeyer et al, 2010). These patients also present with developmental abnormalities implicating a broader role for CBL. As mentioned above, somatic *CBL* mutations are common in MPN and other myeloid malignancies. Interestingly, sporadic JMML cases can also mutate *CBL* somatically which might be equivalent of the germline mutations, but confined to bone marrow tissue (Niemeyer et al, 2010; Sanada et al, 2009).

2. AIMS

The aim of the thesis has been to contribute to the discovery of new germline genetic factors involved in MPN and enhance the knowledge of the mechanisms by which those factors predispose to MPN or influence the pathogenesis. The specific aims were as follows:

- develop a familial linkage analysis algorithm that can easily cope with high-throughput microarray genotyping data;
- use the linkage analysis method for the families with multiple cases of MPN and identify the genomic regions that contain germline causative mutations;
- identify the causative germline mutations in those MPN families by performing whole exome sequencing and focusing on genomic regions showing linkage;
- screen all available familial MPN cases for recurrent germline and somatic mutations the genes with identified germline mutations;
- understand the roles of identified mutated genes in MPN and the pathways they are involved in;
- study the effect of common SNPs on MPN predisposition, as well as in the context of familial MPN, particularly those within the *JAK2* gene region;
- assess the possibility of the interaction of germline variants and somatic genetic changes using SNP microarray analysis and exome sequencing.

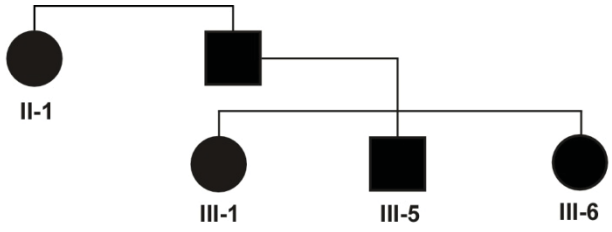
3. RESULTS

3.1 Development of a non-parametric familial linkage analysis algorithm: Segregation exclusion analysis (SEGEX)

The introduction of high throughput genotyping microarrays with dense SNP markers opened up new opportunities for familial linkage analysis. The high number of SNP markers adequately compensates for low polymorphism rate of SNPs and can provide enough power for non-parametric linkage tests. The dramatic increase in SNP density of arrays has not been efficiently utilized in familial linkage analysis and algorithms developed for small number of genotypes cannot cope with massive genotypic data sets due to lack of computational power. The inclusion of many non-polymorphic SNPs creates an additional obstacle for traditional linkage analysis algorithms, as those tend to generate false linkage peaks and cover the real linkage signals. The most commonly used programs currently for non-parametric linkage analysis, GENEHUNTER, dChip, Merlin, Allegro etc., are especially prone to these types of problems since they give different scores when one or both alleles are shared. Another type of scoring proposed by previously (Thomas et al, 1994), which treats single or double allele sharing equally, is less demanding computationally and more robust.

The SEGEX algorithm description. The basic concept of the SEGEX algorithm is to identify all genomic regions not shared among affected members of a pedigree and call genomic regions where such an exclusion of allele sharing is not possible. This method uses only genotypic information from affected members of the family and thus is non-parametric. SNP genotypes of all affected members of a family are listed as a table with each row representing a SNP and each column an affected subject (**Figure 3**). The genotypic calls are presented in form of AA/AB/BB. For each SNP the AAS (absence of allele-sharing) value is assigned as follows: AAS=1 if both AA and BB genotypes are present (absence of allele sharing) among the affected subjects; AAS=0 if allele sharing is present and AA and BB genotypes are not observed together among the affected subjects. After AAS values are assigned to each SNP at a specific physical position (chromosome and position in base pairs), the genomic position for each SNP (x axis) is plotted against its AAS value (y axis) using standard graph plotting software (**Figure 4**). Whenever there is a long genomic

region with the absence of AAS=1 calls, the region can be identified manually as shared among affected subjects of the pedigree. Genomic region >2Mb with the absence of AAS=1 calls were annotated. An example of such a shared region is shown of **Figure 4**.



	II-1	III-1	III-5	III-6	AAS value	Segregation
SNP1	AA	AB	BB	AB	1	excluded
SNP2	AA	AB	AB	AB	0	not excluded
SNP3	AA	AB	AB	BB	1	excluded
⋮	⋮	⋮	⋮	⋮	⋮	
SNPn	AB	BB	AB	AB	0	not excluded

Figure 3. The concept behind SEGEX linkage analysis method.

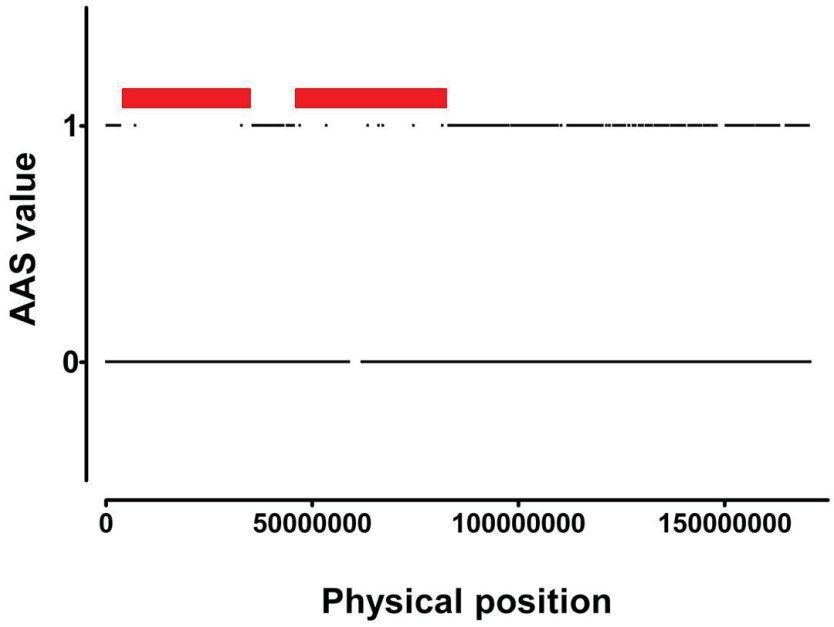


Figure 4. An example of visualization of SEGEX analysis results for one chromosome. The shared regions are marked with red bars.

Frequency of false genotype calls and their influence on the SEGEX algorithm. High-throughput genotyping platforms have a certain error rate. False genotypic calls influence AAS value assignment and can reduce the sensitivity of region calling. To assess the frequency of false genotypes of the SNP microarray platform used in our study we assigned AAS values for a parent-child pair (**Figure 5**).

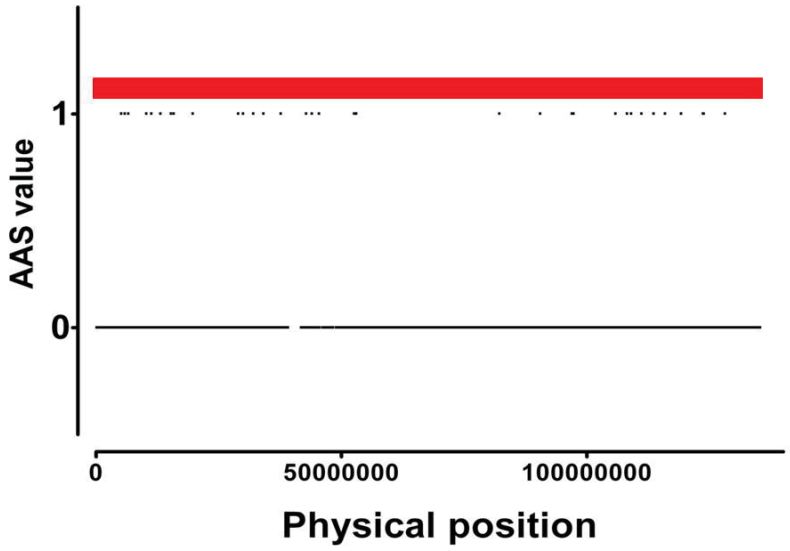


Figure 5. SEGEX results for a parent-child pair showing complete sharing of a chromosome.

As parent and child share one allele for every position of the genome AAS values should equal 0 genome-wide. In the example shown on the Figure 5 1C, few SNPs exhibited AAS=1 in the analyzed parent-child pair. To reduce the effect of wrongly assigned AAS values on shared region calling, the algorithm allows certain number of AAS values of 1 within a long stretch of 0s (as a default we used the threshold of 5 SNPs with AAS=1 within a stretch of 1000 SNPs).

Algorithm validation in a Parkinson's disease pedigree. To validate the SEGEX algorithm we studied a pedigree with Parkinson's disease (PD) in which PD is caused by a previously described *LRRK2* gene mutation R1441C (Zimprich et al, 2004). Six affected members of the family were genotyped using Affymetrix SNP 6.0 microarrays and the shared genomic regions were visualized using the SEGEX algorithm (**Figure 6**). These six affected members of the pedigree represent a total of 10 meioses from the founder (meiotic count=10). As shown in Figure 2a, the vast majority of the genome was excluded and only a single 9.8 Mb region on chromosome 12 was shared among the six affected subjects (**Figure 7**). The *LRRK2*

gene responsible for PD in this pedigree was located within the detected region.

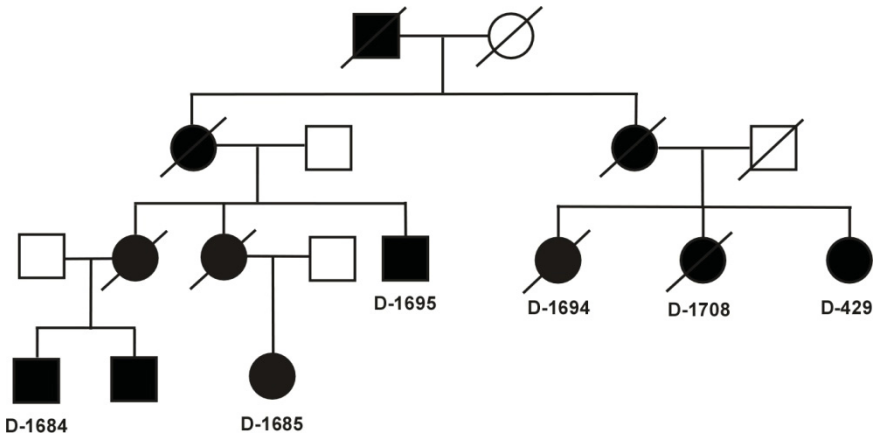


Figure 6. The family tree of Parkinson's disease family used for validation of SEGEX algorithm. The family members marked with sample names were used in the analysis. The affected members are shown as filled circles and squares.

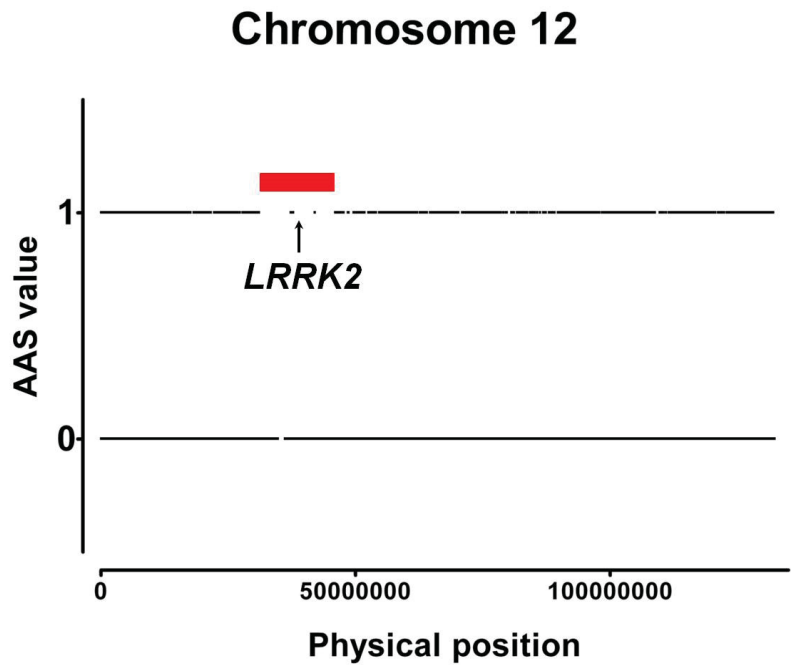


Figure 7. SEGEX results for the Parkinson's disease family. The only genomic region shared by all family members is on chromosome 12 and contains *LRRK2* gene which was shown to carry the pathogenic mutation.

Algorithm validation in two families with hereditary thrombocytosis. We further studied two additional families with known gain-of-function mutations in the *THPO* gene associated with hereditary thrombocytosis (Liu et al, 2008; Wiestner et al, 1998). Hereditary thrombocytosis is an autosomal dominant disorder

characterized by elevated serum thrombopoietin levels and high platelet count. The mutations of the *THPO* gene in hereditary thrombocytosis interfere with the negative translation regulatory sequences in the 5'UTR and result in increased translation efficiency from the *THPO* mRNA. Two unrelated families with hereditary thrombocytosis were included in this study both positive for the G to C transversion in the splice donor of intron 3 of *THPO*. Ten affected members from the first family and eight members from the second family were genotyped. Meiotic counts among the analyzed affected members in these two families were 12 and 8, respectively (**Figure 8**).

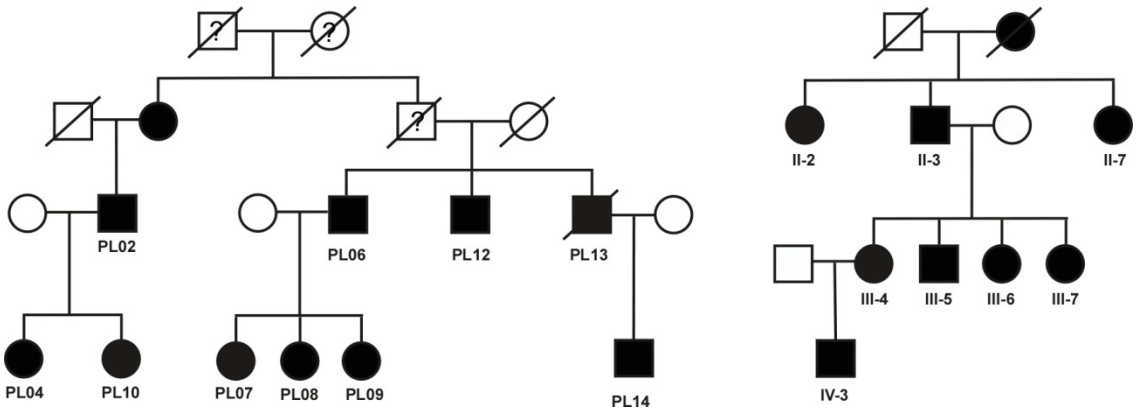


Figure 8. The family trees of the two hereditary thrombocytosis families used for the validation of SEGEX algorithm. The members used in the analysis are marked with samples names.

In the first family the SEGEX algorithm identified a single 10.1 Mb region on chromosome 3 that included the *THPO* gene (**Figure 9**). As the meiotic count and number of affected members was lower in the second family, the SEGEX algorithm detected 3 shared regions; one of them, on chromosome 3, was overlapping with the *THPO* locus (**Figure 10**). The shared regions on chromosome 3 in these two pedigrees overlapped in 7.8 Mb and *THPO* gene was within the minimal overlapping region on chromosome 3.

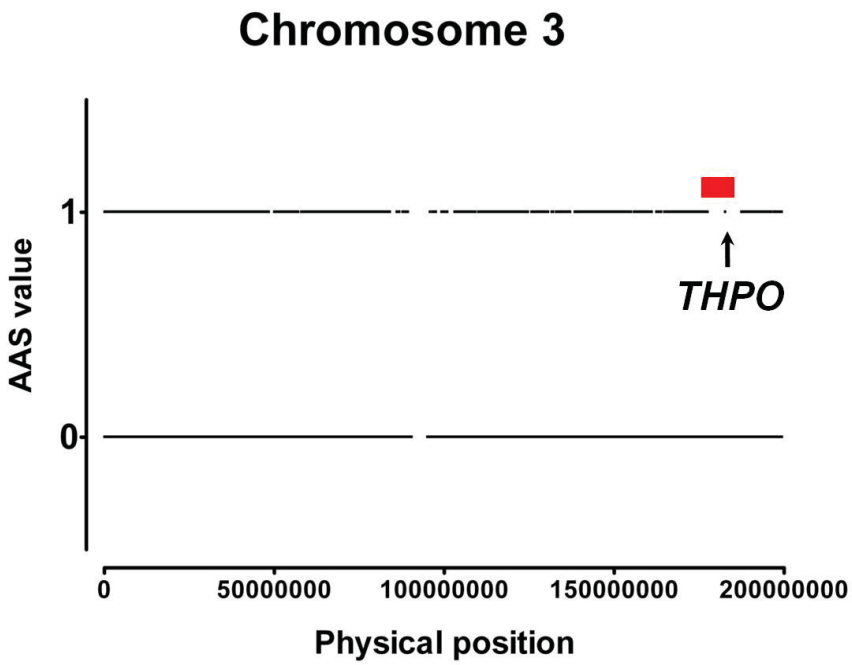


Figure 9. SEGEX results for the first family with hereditary thrombocytosis. The only genomic region shared by all family members is on chromosome 3 and contains *THPO* gene which was shown to carry the pathogenic mutation.

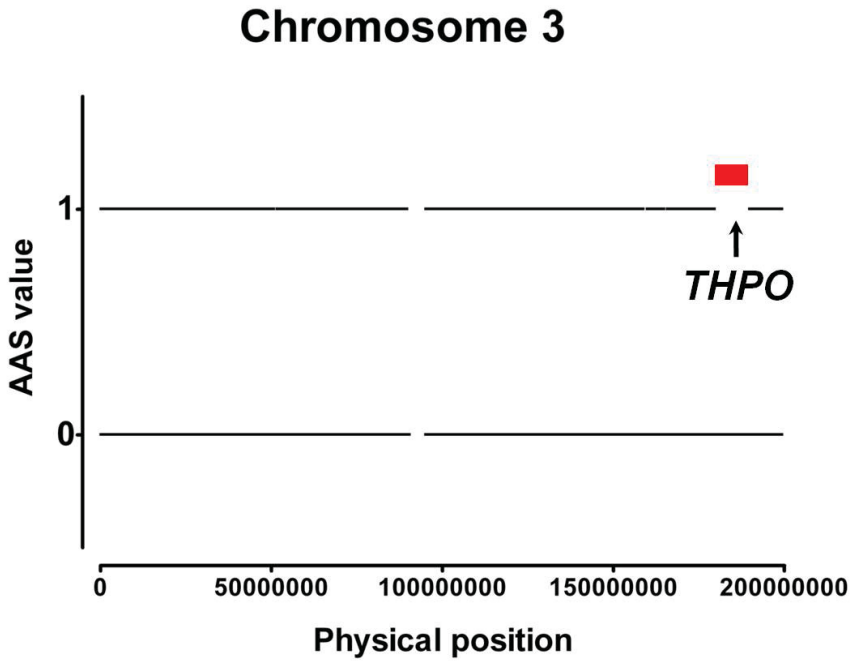


Figure 10. SEGEX results for the second family with hereditary thrombocytosis. One of the three genomic regions shared by all family members is on chromosome 3 and contains *THPO* gene which was shown to carry the pathogenic mutation.

Family size and power of region detection by the SEGEX algorithm. The meiotic count separating the affected individuals included in a SEGEX analysis is

expected to have major influence on the exclusion power of the algorithm. The farther the affected members are within the family the less genomic regions are shared among them. In the most extreme case the whole genome is shared as in case of a parent-child pair (**Figure 5**). When two unrelated individuals are paired and analyzed together no regions are shared. To validate these extremes we have analyzed pairs of randomly selected unrelated subjects. When two unrelated individuals are paired and analyzed together no regions are shared. (**Figure 11**). The unrelated pairs and parent-child pairs represent the two extreme sides of kinship with a close to infinite meiotic count in the former and a meiotic count of one in the latter case.

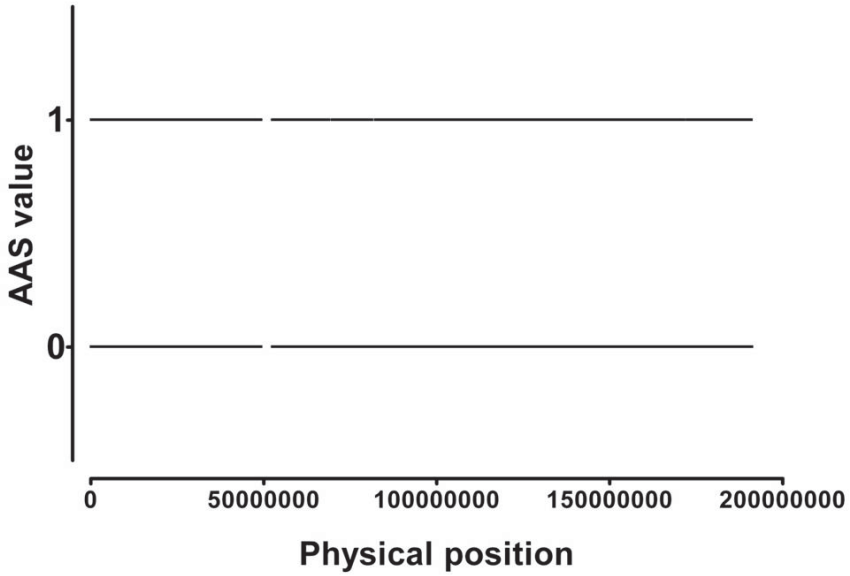


Figure 11. SEGEX results for two unrelated individuals. An example chromosome is shown. No shared regions are detected.

For the remaining cases in between (meiotic counts >1), we examined the relation between meiotic count and the total genomic size of shared genomic regions (in base pairs). The three families used for validation of the SEGEX algorithm were partitioned to obtain new ‘sub-families’ each with a variable meiotic count. The analysis was performed on sub-families and the total size of shared regions was calculated. When meiotic count of each sub-family and the SEGEX detected shared region size is plotted, a negative exponential correlation between meiotic count and size of shared genomic region was observed (**Figure 12**).

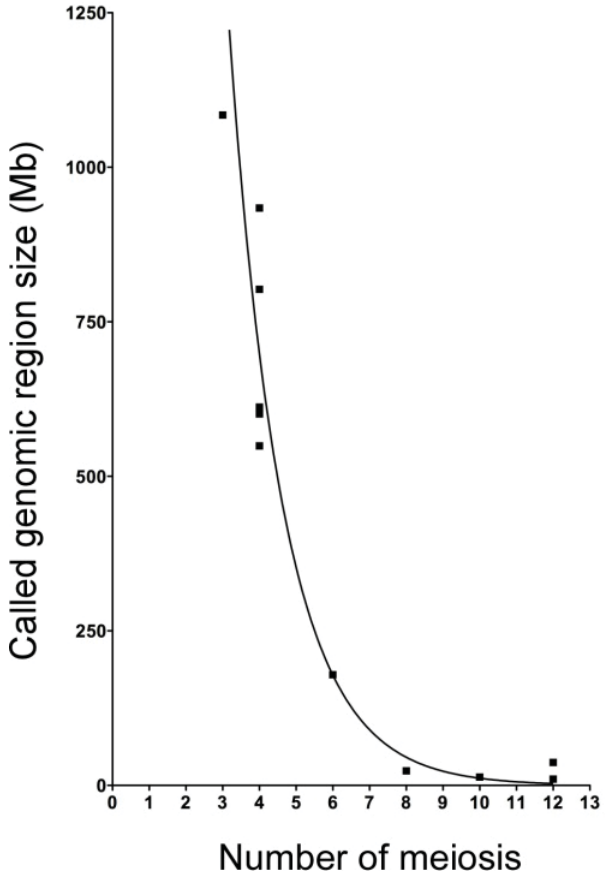


Figure 12. Negative exponential correlation between total size of detected regions by SEGEX and the number of meioses separating all the affected members.

3.2 Identification of germline mutations in *RBBP6* predisposing to myeloproliferative neoplasms

The development of a robust familial linkage analysis algorithm (SEGEX) allowed us to proceed with identification of germline variants predisposing to MPN. In order to identify such a mutation, we studied an Australian MPN pedigree. Five members of the family in four generations have been diagnosed with MPN (**Figure 13**).

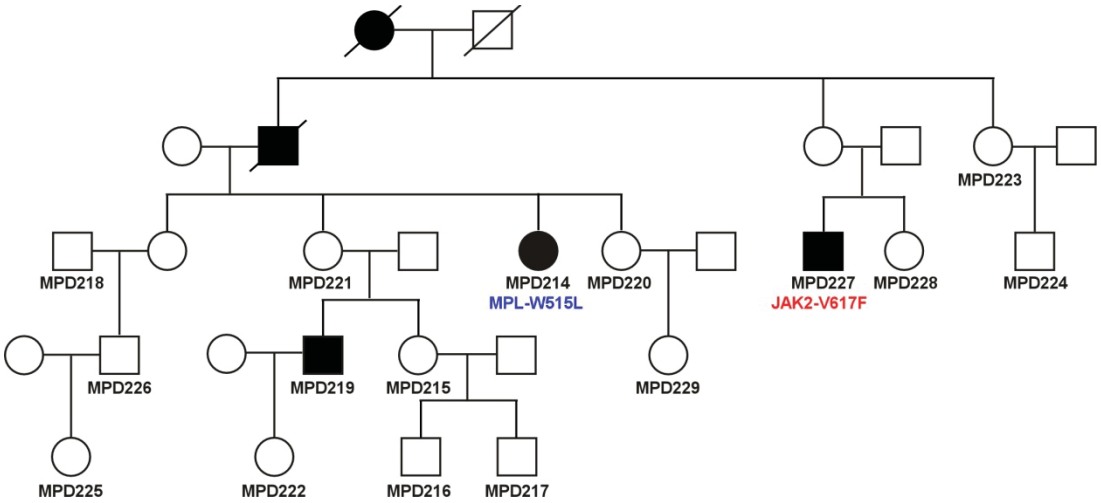


Figure 13. The family tree of the Australian MPN family used in the study. The patients with somatic *JAK2* and *MPL* mutations are marked.

DNA was available from three affected members. One affected member carried JAK2-V617F while another member carried MPL-W515L somatic mutations (**Figure 14**).

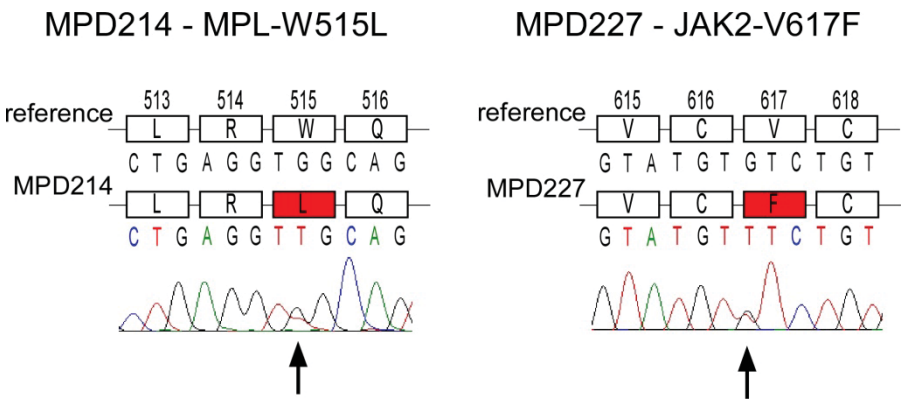


Figure 14. Somatic point mutations in *JAK2* and *MPL* genes detected in affected members of the family.

No *JAK2* or *MPL* mutations were detected in healthy subjects of the pedigree. Two of the three affected subjects carried other somatic lesions detected by SNP microarrays (**Figure 15**).

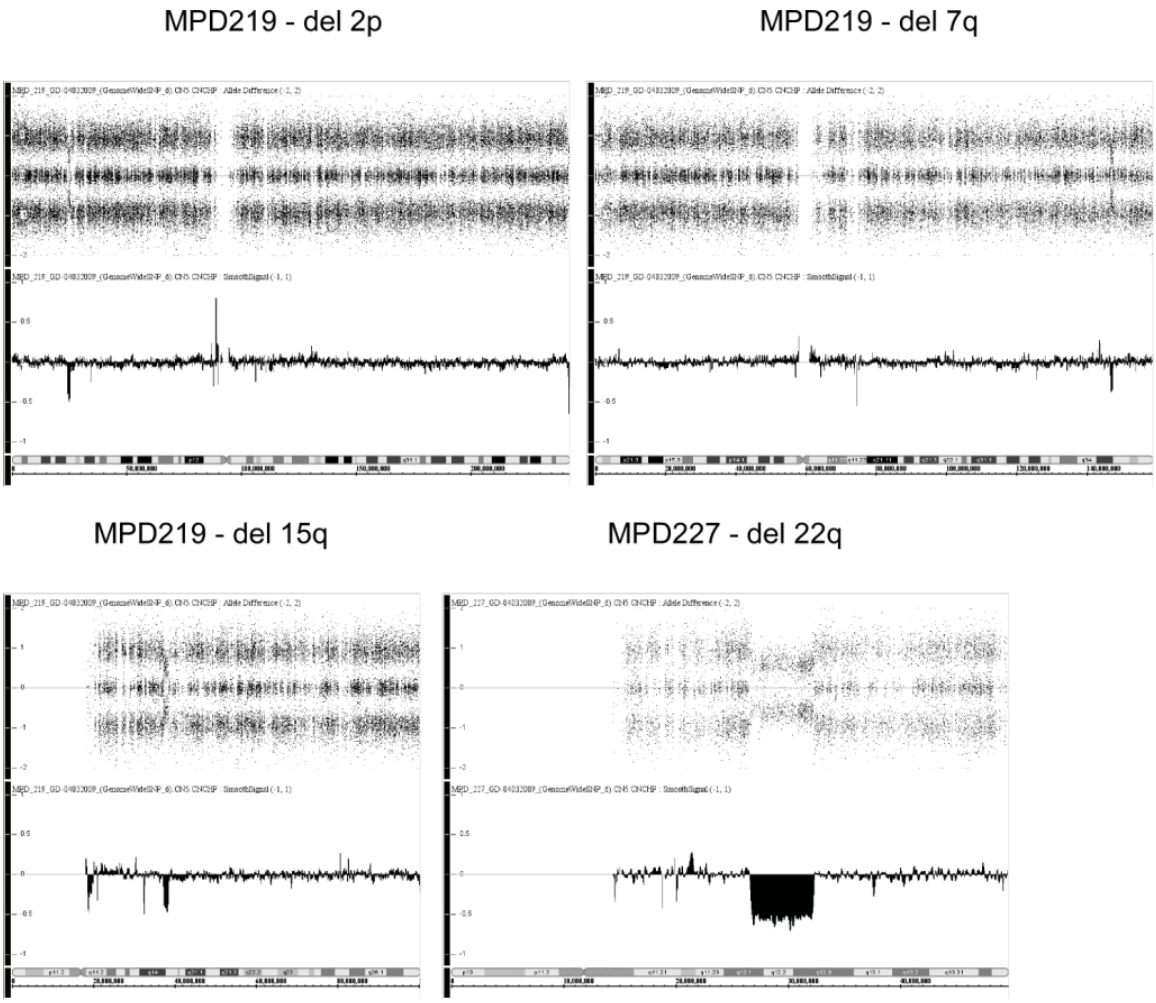


Figure 15. Chromosomal aberrations detected in affected family members by SNP microarrays.

To map the candidate disease loci in the pedigree, we applied our nonparametric algorithm SEGEX. Using about 900,000 SNP genotypes, we identified 12 shared genomic regions with a total size of 217.87 Mb amongst the three affected subjects (**Figure 16, Table 2**).

Table 2. Shared genomic regions detected in the family by SEGEX analysis.

Chromosome	Start	End	Size, Mb
3	169957402	183120944	13.16
4	126496421	179602914	53.11
4	185211312	191167888	5.96
6	18820505	23795069	4.97
13	23737574	35954484	12.22
14	99164870	106356482	7.19
16	14336994	35063218	20.73
17	52326240	67187833	14.86
20	9895067	24959423	15.06
20	38969553	50664093	11.69
X	109805	13612919	13.50
X	46406761	91827344	45.42

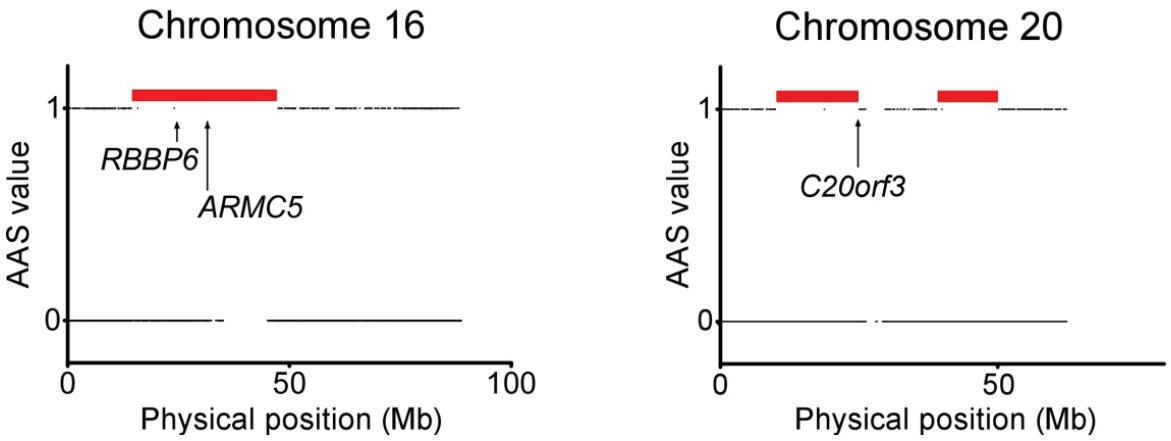


Figure 16. Genomic regions shared by the three affected members in the family identified by the SEGEX analysis. Arrows indicate the physical position of the the candidate genes *RBBP6*, *ARMC5*, and *C20orf3*.

As one of these genomic regions was likely to carry the disease-causing mutation, we applied next generation exome sequencing. After reference alignment, single nucleotide variants (SNV) and deletion-insertion variants (DIV) were detected and a number of filtering criteria were applied (**Figure 17**).

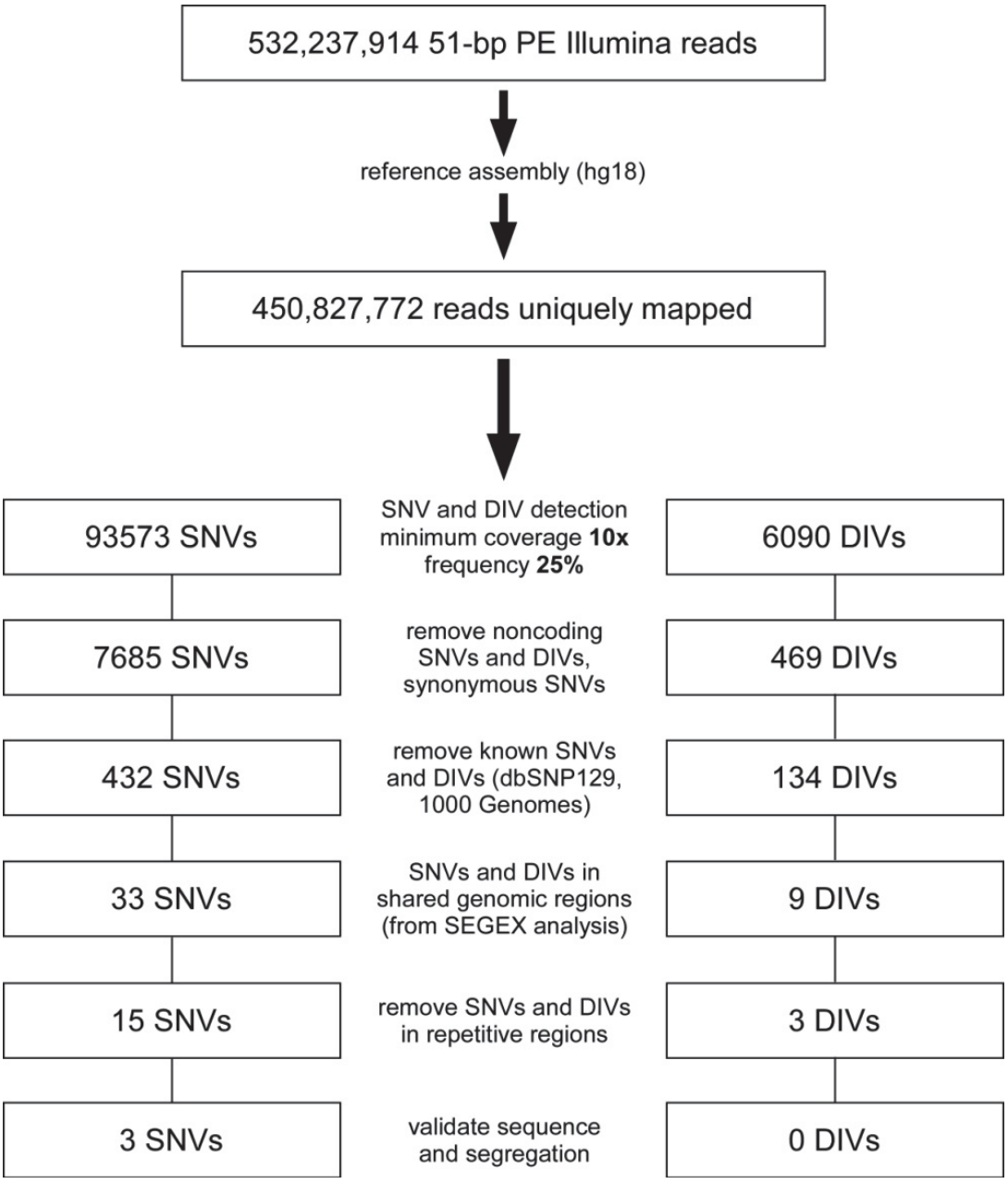


Figure 17. Exome sequencing data analysis and variant filtering for the Australian family

We performed Sanger sequencing of all the 18 final candidate variants and confirmed DNA variants segregating with the disease in three genes (*RBBP6*, *C20orf3*, and *ARMC5*) (**Figure 18, Table 3**).

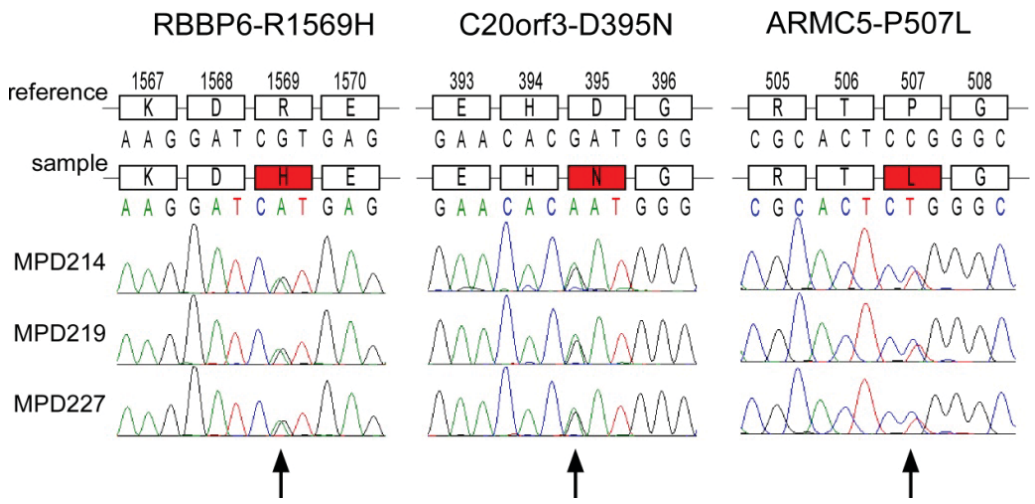


Figure 18. Validation of the mutations in *RBBP6*, *ARMC5*, and *C20orf3* segregating with the disease in the pedigree. The locations of mutations are marked with arrow.

Table 3. Validation of detected SNVs and DIVs from exome sequencing of the Australian family.

						Genotypes			
chr	position	DNA change	mutation type	amino acid change	gene	MPD_214	MPD_219	MPD_227	Validation
4	146252857	C>G	missense	P243A	*ABCE1*	C/C	C/C	C/C	No
4	177842076	delTCA	deletion	M418del	*VEGFC*	---/---	---/---	---/---	No
4	191110341	A>G	missense	E55G	*FRG1*	A/A	A/A	A/A	No
13	23919323	A>G	missense	I1039T	*PARP4*	A/A	A/A	A/A	No
13	35910866	delT	deletion	V252GfsX29	*CCNA1*	T	T	T	No
14	104492040	G>A	nonsense	Q265X	*AHNAK2*	G/G	G/G	G/G	No
16	15692679	G>A	missense	R234H	*NDE1*	G/A	G/A	G/G	No
16	24490594	G>A	missense	**R1569H**	***RBBP6***	G/A	G/A	G/A	**Yes**
16	31383365	C>T	missense	**P507L**	***ARMC5***	C/T	C/T	C/T	**Yes**
17	62457148	T>G	missense	S184A	*CACNG4*	T/T	T/T	T/T	No
20	24892517	G>A	missense	**D395N**	***C20orf3***	C/T	C/T	C/T	**Yes**
20	43437570	delATT	deletion	N97del	*TP53TG5*	ATT/---	ATT/---	ATT/ATT	No
20	44607932	G>T	missense	T163N	*C20orf123*	G/G	G/G	G/G	No
20	48654710	T>C	missense	D318G	*FAM65C*	C/T	C/C	C/T	No
X	2842668	A>G	stop loss	X383Q	*ARSD*	A/A	A/A	A/A	No
X	57635595	G>A	missense	G130D	*ZXDB*	G/G	G/G	G/G	No
X	66683162	C>A	nonsense	Y483X	*AR*	C/C	C/C	C/C	No
X	11693616	G>T	missense	A174S	*MSL3L1*	G/T	G/G	G/G	No

Abbreviations: chr, chromosome; del, deletion; fs, frameshift.

To identify which of the three variants is causative, we applied several strategies. Firstly, we examined healthy subjects for the presence of the candidate variants (**Table 4**). Based on this analysis, ARMC5-P507L was excluded due to 7% frequency in controls while RBBP6-R1569H and C20orf3-D395N were not found in any of over 700 controls.

Table 4. Comparison of three validated and segregating variants detected in Australian family.

gene	cDNA change	protein change	PolyPhen2 score	SIFT score	Phast conservation	in healthy controls
RBBP6	c.4706G>T	R1569H	0.766*	0.04^{+}	0.990	0/715
C20orf3	c.1183G>A	D395N	0.002	0.53	0.834	0/701
ARMC5	c.1520C>T	P507L	0.001	0.03^{+}	0.000	5/71

*possibly damaging, $^{+}$damaging.

Abbreviations: cDNA, complementary deoxyribonucleic acid; c., cDNA position.

Next we sequenced the exons carrying the *RBBP6* and *C20orf3* mutations in an additional 66 MPN families. This analysis yielded two unique mutations in *RBBP6* (E1654G and R1451T) (**Figure 19**, **Table 5**) and one polymorphism in *C20orf3* (P406L) present in 4% of the controls.

Table 5. Summary of *RBBP6* variants in familial and sporadic MPN cases.

Pedigree	Sample	diagnosis	JAK2/MPL	cDNA change	amino acid change	Polyphen 2 score	in healthy controls
1	MPD214	ET	MPL-W515L	c.4706G>T	R1569H	0.766*	0/715
	MPD219	PMF	-	c.4706G>T	R1569H		
	MPD227	PMF	JAK2-V617F	c.4706G>T	R1569H		
2	f16p1	PMF	JAK2-V617F	c.4961A>G	E1654G	0.375	0/649
3	570	PMF	JAK2-V617F	c.4352G>C	R1451T	0.942**	0/642
4	MPC07-350	ET	JAK2-V617F	c.5332_5337del	K1778-E1779del	N/A	29/202
Sporadic	H_0327	PV	JAK2-Ex12del	c.4331C>T	S1444F	0.976**	0/650
Sporadic	H_0580	ET	JAK2-V617F	c.4331C>T	S1444F	0.976**	0/650
Sporadic	H_0465	ET	-	c.4981A>G	I1661V	0.001	3/649
Sporadic	H_0437	PV	-	c.5018C>T	A1673V	0.010	0/607

*possibly damaging, **probably damaging.

Abbreviations: Ex12del – exon 12 deletion E543-D544; del, deletion; N/A, not available.

In summary, we identified three new *RBBP6* mutations in a total of 67 MPN pedigrees (4.5%). Apart from the three affected subjects eight additional healthy subjects carried RBBP6-R1569H in the Australian pedigree, consistent with the expected low penetrance and in agreement with previous estimates (Olcaydu et al, 2011). Due to the low penetrance associated with *RBBP6* mutations, establishment of family history of MPN may be difficult. Therefore, we screened for *RBBP6* mutations in 490 sporadic MPN cases. In this analysis we identified two unique

germline mutations (S1444F and A1673V) in three apparently unrelated patients and one polymorphism (I1661V) present in 0.5% of controls (**Table 5**; **Figure 19**). Overall, we identified 5 different germline *RBBP6* mutations associated with MPN and not detected in the general population (**Table 5**).

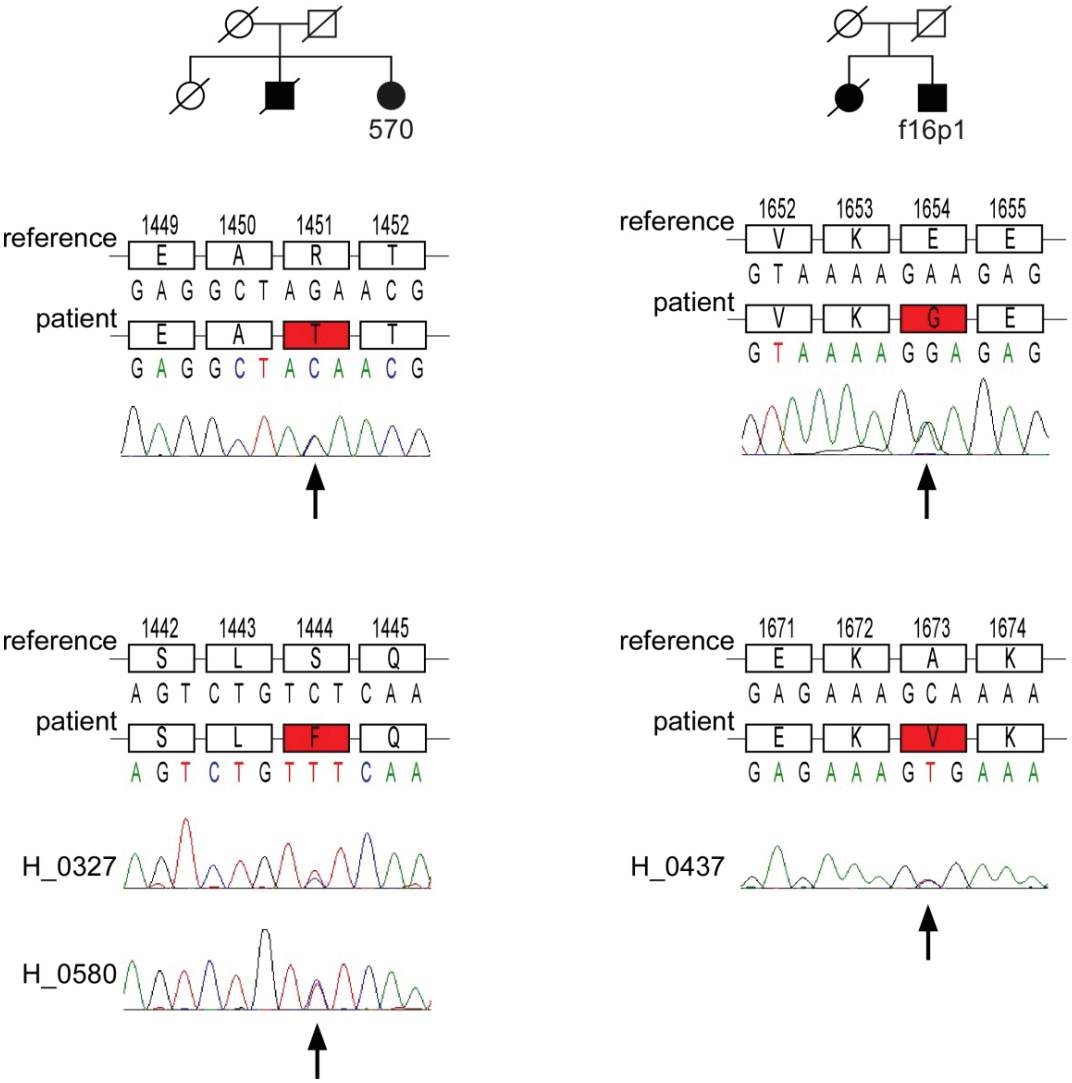


Figure 19. The germline *RBBP6* mutations found in familial and sporadic MPN cases. The family trees with their respective mutations are shown on top, while the mutations in sporadic MPN cases are shown at the bottom of the figure.

Several previous reports have shown that RBBP6 is a negative regulator of p53, has putative E3 ubiquitin ligase activity and predicted Rb and p53 binding regions (UniProtKB, /www.uniprot.org/)(Li et al, 2007; Simons et al, 1997). Together with Mdm2, Rbbp6 ubiquitinates and degrades p53 (Li et al, 2007). As the *RBBP6* mutations identified in this study were all located in the vicinity of the p53-binding domain, we hypothesized that *RBBP6* mutations may affect p53 function.

Interestingly, all the *RBBP6* mutations identified in our study are clustered in the p53-binding domain (**Figure 20**).

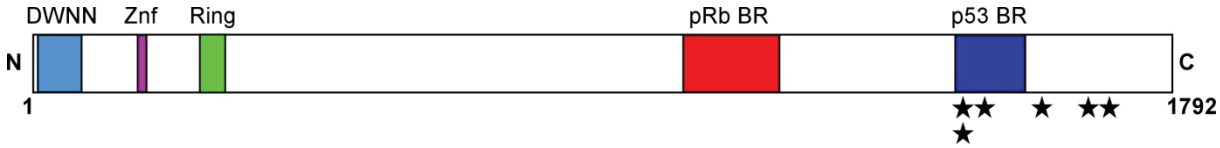


Figure 20. The localization of RBBP6 mutations. The schematic structure of RBBP6 protein with known and predicted domains is shown. The locations of the detected mutations that are not observed in healthy controls are marked with stars. BR, binding region; Znf, zinc finger domain; DWNN, domain with no name.

3.3 Manuscript # 1: p53 lesions in leukemic transformation

Harutyunyan A. et al.,

published in ***N Engl J Med*** 2011; 364: 488-490. Reprinted with permission.

There is accumulating evidence that p53 plays an important role in the pathogenesis of myeloproliferative neoplasms (Beer et al, 2010a; Nakatake et al, 2012). We conducted a SNP microarray study of more than 400 MPN patients in different phases of the disease (Klampfl et al, 2011). The study revealed a number of recurrent chromosomal aberrations, among which were gains in the long arm of the chromosome 1.

The minimal common amplified region on chromosome 1q spanned about 3.5 Mb and contained *MDM4* gene, a known negative regulator of p53. This aberration was particularly frequent in the stage of leukemic transformation of the patients. This finding led us to screen for *TP53* mutations in MPN patients that transformed to sAML. We found six patients with *TP53* mutations in the group of 19 patients with leukemic transformation (Harutyunyan et al, 2011b). All the mutations were somatically acquired. Interestingly, the *TP53* mutations and gains of chromosome 1q were mutually exclusive, which is in agreement with similar reports in other cancers (Veerakumarasivam et al, 2008).

The frequency of *TP53* mutations in sAML group was significantly higher than in the chronic phase MPN. *TP53* mutations combined with gains of chromosome 1q are present in about 45% of post-MPN AML patients (Harutyunyan et al, 2011b). This finding establishes p53 as one of the key pathways involved in the leukemic transformation of MPN patients. However, p53 pathway seems to be involved also in the pathogenesis of chronic phase MPN (Nakatake et al, 2012) and in some cases of germline predisposition (our data on *RBBP6* mutations, see section 3.2).

In the paper I have performed the analysis of microarray data, Sanger sequencing of *TP53* in sAML and chronic phase MPN patients. I wrote the paper together with my supervisor, Dr. Robert Kralovics.

p53 Lesions in Leukemic Transformation

TO THE EDITOR: Myeloproliferative neoplasms have an inherent tendency toward leukemic transformation. The genetic mechanisms of transformation remain largely unknown. We analyzed biopsy specimens of myeloproliferative neoplastic tissue from 330 patients for chromosomal aberrations associated with leukemic transformation (the analysis was performed with the use of Genome-Wide Human SNP [single-nucleotide polymorphism] Array, Affymetrix). Of those patients, 308 had chronic-phase myeloproliferative neoplasms and 22 had postmyeloproliferative-phase neoplasm secondary acute myeloid leukemia (AML). Among these 22 patients, 1 carried the myeloproliferative leukemia virus oncogene (*MPL*) W515L and all others carried the Janus kinase 2 gene (*JAK2*) V617F mutation. Amplifications of chromosome 1q were significantly associated with transformation to AML (0.32% in patients with chronic-phase myeloproliferative neoplasms and 18.18% in patients with secondary AML; P<0.001). The minimal amplified region on chromosome 1q (201.0 to 204.5 Mbp) harbored *MDM4* (Fig. 1A), a potent inhibitor of p53 often amplified in several types of cancer.[1] This observation led us to investigate the involvement of the p53 pathway in postmyeloproliferative-neoplasm AML.

We sequenced the *TP53* gene from all patients in whom leukemic transformation had occurred and found that 6 patients (27.3%) carried somatic mutations. Three of the patients had indepen-

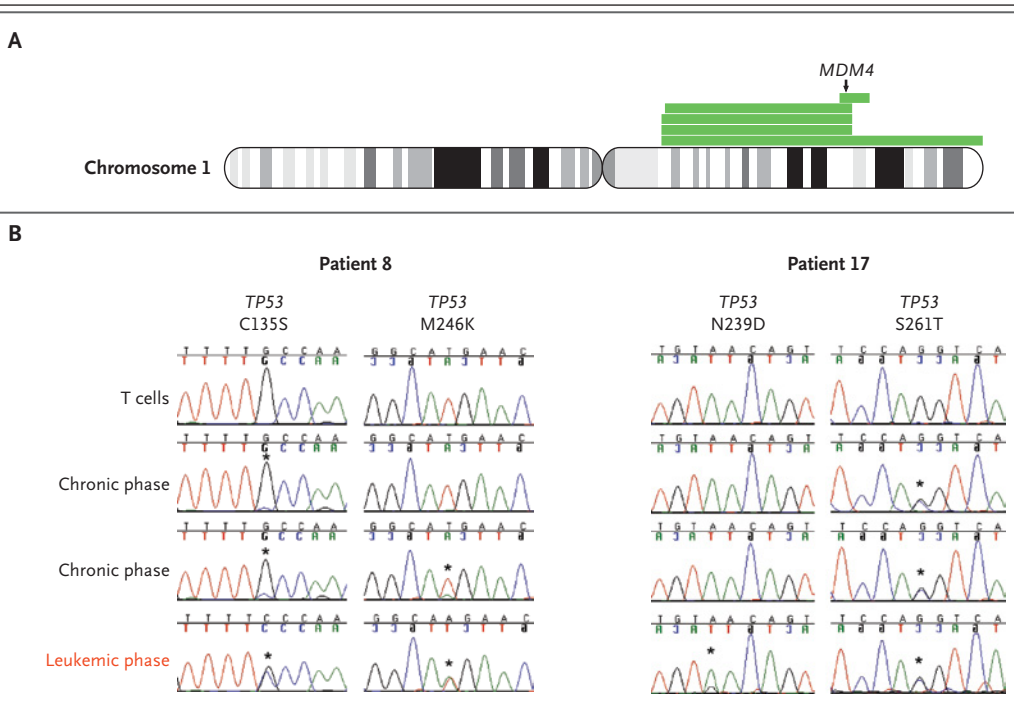


Figure 1. Amplifications of Chromosome 1q and *TP53* Mutations in Patients with Postmyeloproliferative-Neoplasm Acute Myeloid Leukemia.

In Panel A, the green bars indicate regions of recurrent amplifications on chromosome 1q. The minimal amplified region on chromosome 1q contains the *MDM4* gene. Panel B shows the results of an analysis of malignant tissue samples (granulocytes) at different time points from two patients in whom there was a transformation to postmyeloproliferative-neoplasm acute myeloid leukemia; both also harbored biallelic *TP53* missense mutations. T cells have been used as a control tissue. The asterisks show the position of the mutations. The antisense direction of the sequencing is indicated by reverse lettering.

Table 1. Summary of *TP53* Mutations and Other Lesions in Patients with Postmyeloproliferative-Neoplasm Acute Myeloid Leukemia.

Patient No.	*JAK2*	*MPL*	Gains of Chromosome 1q	*TP53*	*IDH1*	*IDH2*	*LNK*
1	V617F	Wild type	—	Wild type	Wild type	Wild type	Wild type
2	V617F	Wild type	—	Wild type	Wild type	Wild type	Wild type
3	V617F	Wild type	—	Wild type	Wild type	R140W	Wild type
4	V617F	Wild type	Yes	Wild type	Wild type	Wild type	Wild type
5	V617F	Wild type	—	Wild type	Wild type	Wild type	Wild type
6	V617F	Wild type	—	c.994-2 A>G (17pUPD)	Wild type	Wild type	Wild type
7	V617F	Wild type	Yes	Wild type	Wild type	Wild type	Wild type
8	V617F	Wild type	—	C135S/M246K	Wild type	Wild type	Wild type
9	V617F	Wild type	—	Wild type	Wild type	Wild type	Wild type
10	V617F	Wild type	Yes	Wild type	Wild type	Wild type	Wild type
11	V617F	Wild type	—	Wild type	Wild type	Wild type	Wild type
12	V617F	Wild type	—	Wild type	Wild type	Wild type	Wild type
13	V617F	Wild type	—	N239D (17pUPD)	Wild type	Wild type	Wild type
14	V617F	Wild type	—	c.560-1 G>A/Y220H	Wild type	Wild type	Wild type
15	V617F	Wild type	—	Wild type	Wild type	Wild type	Wild type
16	V617F	Wild type	—	K132E	Wild type	Wild type	Wild type
17	V617F	Wild type	—	S261T/N239D	Wild type	Wild type	Wild type
18	V617F	Wild type	—	Wild type	Wild type	Wild type	Wild type
19	V617F	Wild type	—	Wild type	Wild type	Wild type	Wild type
20	V617F	Wild type	—	Wild type	Wild type	Wild type	Wild type
21	V617F	Wild type	—	Wild type	R132G	Wild type	Wild type
22	Wild type	W515L	Yes	Wild type	Wild type	Wild type	Wild type

* UPD denotes uniparental disomy.

dent mutations on both *TP53* alleles, and 2 had homozygous mutations because of an acquired uniparental disomy of chromosome 17p. One patient had only one mutated *TP53* allele (Table 1). None of the patients with *TP53* mutations had amplification of chromosome 1q. The phenomenon of mutual exclusivity of *TP53* mutations and *MDM4* amplifications has been also observed in solid tumors.[2]

Among the 22 patients with postmyeloproliferative-neoplasm AML, 10 (45.5%) had evidence of a p53-related defect mediated by *TP53* gene mutations or gains of chromosome 1q (Table 1). We detected monoallelic *TP53* mutations (R283C and E298K) in 2 of 65 patients with chronic-phase myeloproliferative neoplasms, indicating that low mutation frequency is associated with this condition (3.1%). Thus, in our cohort, *TP53* mutations were strongly associated with transformation to AML in patients with myeloproliferative neoplasms ($P=0.003$). Recent reports have implicated *IDH1/2*, *LNK*, and *IKZF1* in this transformation.[3-5] We found one mutation in *IDH1* and one in *IDH2* in postmyeloproliferative-neoplasm AML but no *LNK* mutations (Table 1). In our cohort, *TP53* mutations and 1q gains were the most frequent lesions associated with postmyeloproliferative-neoplasm AML.

Tissue samples from chronic-phase myeloproliferative neoplasms were available from two of the patients who carried biallelic *TP53* mutations and whose condition had progressed to secondary AML. Patient 8 carried both *TP53* mutations in the chronic phase, but in a smaller clone; in Patient 17, only one of the two mutations was present in the chronic phase (Fig. 1B). The fact

that *TP53* mutations were detectable in both patients during the chronic phase suggests that *TP53* mutations may predict leukemic transformation in myeloproliferative neoplasms.

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Disclosure forms provided by the authors are available with the full text of this letter at NEJM.org.

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Letters to the Editor are considered for publication, subject to editing and abridgment, provided they do not contain material that has been submitted or published elsewhere. Please note the following:

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CORRECTIONS

Telemonitoring in Patients with Heart Failure (December 9, 2010;363:2301-9). In Table 1 (page 2305), the data for white race were incorrect. In the Telemonitoring column, the number of patients should have been 414, rather than 413, and in the Usual Care column, the number and percentage should have been 401 (48.5), rather than 402 (48.6). The article is correct at NEJM.org.

Tourette's Syndrome (December 9, 2010;363:2332-8). In the final paragraph (page 2337), the third to last sentence, beginning "For combined . . . ," should have ended, ". . . although this agent is not approved by the FDA for Tourette's syndrome," rather than ". . . although this agent is not approved by the FDA for these conditions." We regret the error. The article is correct at NEJM.org.

Management of Varices and Variceal Hemorrhage in Cirrhosis (March 4, 2010;362:823-32). Dr. Bosch's affiliation (page 823) should have been "Hospital Clinic, Hepatic Hemodynamic Laboratory, Liver Unit, University of Barcelona, and Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas — both in Barcelona." Also, in Table 3 (page 828), the dose for somatostatin should have been "Intravenous 250-μg bolus, followed by infusion of 250-500 μg/hr," rather than "Intravenous 250-mg bolus, followed by infusion of 250 mg/hr." The article is correct at NEJM.org.

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The conference will be held in Zurich, Switzerland, Aug. 17–20. It is organized by the European Society for Philosophy of Medicine and Healthcare (ESPMH) and the Institute of Biomedical Ethics, University of Zurich, Switzerland. Deadline for submission of abstracts is March 1.

Contact Prof. Bert Gordijn, Secretary of the ESPMH, Institute of Ethics, Henry Grattan Building, Dublin City University, Dublin 9, Ireland; or e-mail bert.gordijn@dcu.ie.

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3.4 Manuscript # 2: A Common *JAK2* haplotype confers susceptibility to myeloproliferative neoplasms

Olcaydu D., Harutyunyan A. et al.

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More than 95% of patients with PV and 50-60% of ET and PMF patients have acquired mutations in *JAK2*. Additional chromosomal aberrations and point mutations are acquired during the course of the disease, generating distinct subclones. Due to long duration of the disease, often a number of mutations and chromosomal aberrations create a complex clonal structure in MPN patients (Kralovics, 2008).

We investigated the clonal structure in MPN patients by growing and genotyping single progenitor colonies for detected mutations in each patient. In some patients there was evidence of independent acquisition of JAK2-V617F mutation in different subclones. Following up on this finding, we developed a PCR-based assay that detects the relationship of V617F mutation and a nearby germline polymorphism, rs12343867 in patients heterozygous for that SNP (Olcaydu et al, 2009a).to screen for multiple acquisition the mutation. We found 3 cases of multiple acquisition of JAK2-V617F in a cohort of 109 MPN patients. Interestingly, there was a non-random pattern of V617F mutation acquisition: the mutation happened significantly more often on the chromosome carrying C allele of the SNP than on the one with T allele (Olcaydu et al, 2009a). Considering this finding in the context of a recent report that SNPs in *JAK2* region are differentially distributed in PV and ET patients (Pardanani et al, 2008), we hypothesized that the JAK2 haplotype confers risk to acquire JAK2-V617F mutation and thus to develop MPN. It was confirmed by genotyping MPN patients and healthy controls for the rs12343867 and surrounding SNPs. Further analyses delineated the *JAK2* haplotype, termed 'GGCC', and showed that this haplotype increases the risk to develop JAK2-V617F positive MPN several fold (Olcaydu et al, 2009a), but does not confer increased risk of JAK2-negative MPN.

In this paper I have been involved in genotyping cases and controls for *JAK2* SNPs, statistical analysis of the data, as well as writing the paper together with first author Damla Olcaydu and my supervisor Dr. Robert Kralovics.

nature genetics

A common *JAK2* haplotype confers susceptibility to myeloproliferative neoplasms

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Genome-wide association studies have identified a number of new disease susceptibility loci that represent haplotypes defined by numerous SNPs. SNPs within a disease-associated haplotype are thought to influence either the expression of genes or the sequence of the proteins they encode. In a series of investigations of the *JAK2* gene in myeloproliferative neoplasms, we uncovered a new property of haplotypes that can explain their disease association. We observed a nonrandom distribution of the somatic *JAK2*^{V617F} oncogenic mutation between two parental alleles of the *JAK2* gene. We identified a haplotype that preferentially acquires *JAK2*^{V617F} and confers susceptibility to myeloproliferative neoplasms. One interpretation of our results is that a certain combination of SNPs may render haplotypes differentially susceptible to somatic mutagenesis. Thus, disease susceptibility loci may harbor somatic mutations that have a role in disease pathogenesis.

Myeloproliferative neoplasms (MPN) are blood diseases characterized by clonal hematopoiesis, chronic excessive production of differentiated blood cells and increased risk for thrombosis and secondary leukemic transformation. Three major diseases constitute MPN: polycythemia vera, essential thrombocythemia and primary myelofibrosis. A somatic gain-of-function mutation of the *JAK2* kinase gene on chromosome 9 has been identified in more than 50% of individuals with MPN[1–4]. The mutation is an invariant G-to-T transversion in exon 14 resulting in a valine-to-phenylalanine substitution in codon 617 (*JAK2*^{V617F}). Only rare cases of additional mutations are found in MPN, including mutations in exon 12 of *JAK2* and exon 10 of *MPL* genes[5–7]. In addition to these oncogenic mutations, chromosomal aberrations are also frequent in MPN. Although a recent study suggested that the observed chromosomal instability in MPN is dependent on *JAK2*^{V617F}, other studies have shown occurrence of chromosomal aberrations outside the *JAK2*^{V617F}-positive clone[8,9]. Thus, the origin of chromosomal instability in MPN remains unclear and it has been hypothesized that mutations acquired before *JAK2*^{V617F} might be responsible[10].

In our search for cytogenetic aberrations that precede *JAK2*^{V617F}, we identified an individual with primary myelofibrosis positive for *JAK2*^{V617F} and with a complex karyotype. The individual had deletions on chromosomes 13q (del13q) and 20q (del20q) and an acquired uniparental disomy of chromosome 9p (**Fig. 1**). To examine the clonal relation of *JAK2*^{V617F} with chromosomal aberrations, we genotyped individual hematopoietic progenitor clones for the presence of *JAK2*^{V617F}, del13q and del20q. Using these three clonality markers, six genotypic classes (A–F) of progenitors were identified (**Fig. 1a**). The most numerous progenitor clone (E) showed homozygosity for *JAK2*^{V617F} and was positive for del13q. We observed colonies single positive for del13q (D) and for *JAK2*^{V617F} in a heterozygous state (B). To draw a conclusion on the clonal hierarchy of progenitors with this distribution of genotypes, we had to postulate that there were two independently acquired *JAK2*^{V617F} mutations, one occurring on wild type and the other on a del13q-positive background (**Fig. 1b**). Deletion on chromosome 20q was acquired on a homozygous *JAK2*^{V617F} background that was negative for del13q. Using microarray analysis, we could confirm the genotypic classes of progenitors (**Fig. 1c**).

The most notable conclusion of our cytogenetic and clonal hierarchy analyses was the evidence for multiple acquisitions of *JAK2*^{V617F} in the same individual. To assess the frequency of multiple acquisition of *JAK2*^{V617F} among individuals with MPN, we designed an assay using a SNP in intron 14 (rs12343867) of *JAK2* 418 bp away from the *JAK2*^{V617F} mutation in exon 14 (**Fig. 2a,b**). Heterozygosity for rs12343867 distinguishes which of the two alleles of *JAK2* acquired *JAK2*^{V617F} (**Fig. 2a**). Allele-specific PCR using a primer specific to *JAK2*^{V617F} and a primer distal to rs12343867 generates PCR products that can be genotyped with a Taqman assay for rs12343867. 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 (**Fig. 2b**). Of our *JAK2*^{V617F}-positive subjects with MPN ($n = 213$), 109 were heterozygous for rs12343867 and thus informative for the assay. Although most subjects carried the mutation on only one of the two alleles, we detected three subjects (2.8%) who acquired *JAK2*^{V617F} on two *JAK2* alleles (**Fig. 2c**). This was confirmed by subcloning of the PCR product and sequencing (**Fig. 2d**). Our assay captures only part of all cases with two *JAK2*^{V617F}-positive clones

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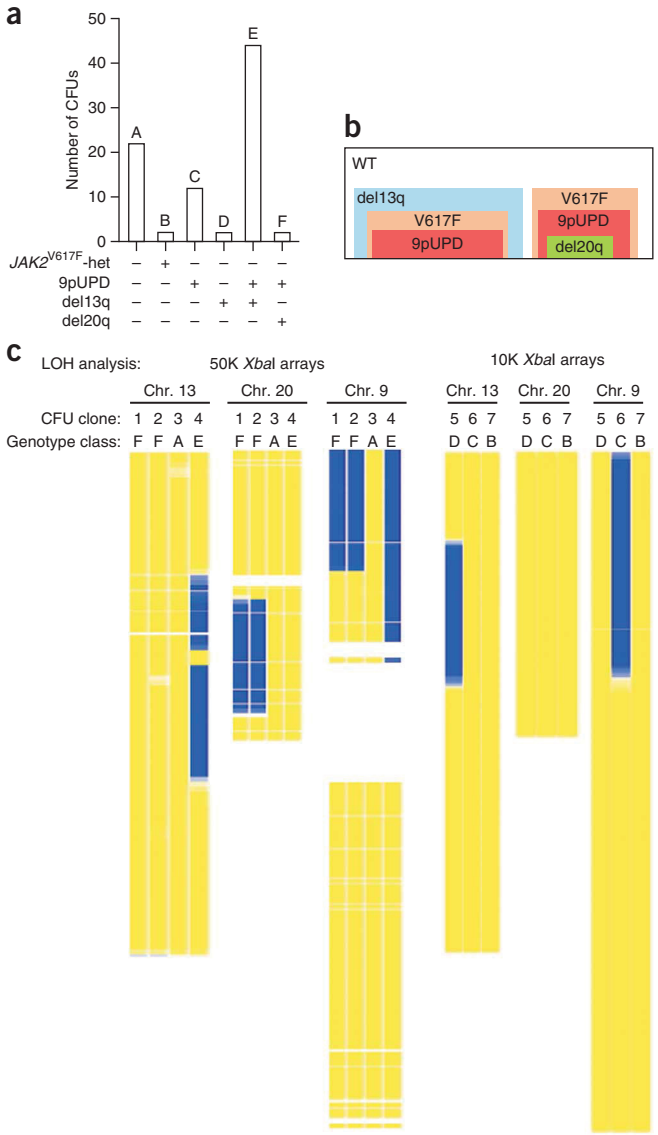

Figure 1 Cytogenetic analysis of hematopoietic progenitor clones in a subject with primary myelofibrosis. (**a**) Genotyping of colony forming units (CFU; primarily consisting of erythroid burst-forming units and granulocytic-monocytic CFUs) for the presence of *JAK2*^{V617F} (het, heterozygous), uniparental 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 subject (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 (loss of heterozygosity (LOH) plots are not in scale between the two array types). We detected LOH using the dChip software. Only chromosomes 13, 20 and 9 (chr. 13, chr. 20, chr. 9) 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 position in clones 1,2 (genotypic class F) and 4,6 (genotypic classes C,E), respectively.

because we cannot detect cases in which the two independent *JAK2*^{V617F} mutations occurred on the same allele (**Fig. 2a**). Thus, the actual frequency of subjects with at least two *JAK2*^{V617F} mutations among the 109 informative cases could be higher than 2.8%. Multiple acquisitions of oncogenic mutations in the same gene have not been previously demonstrated in other hematological malignancies, but multiple *KRAS* and multiple *KIT* mutations were found in several types of solid tumors[11–15]. A previously published report showed coexistence of *JAK2*^{V617F} with a *JAK2* exon 12 deletion in the same individual in independent clones[16]. This report and our results indicate that individuals with MPN 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 two parental alleles. However, with the *JAK2*^{V617F} oncogene, we observed an unequal distribution. The vast majority of subjects (93/109; 85%) heterozygous for rs12343867 carried the *JAK2*^{V617F} mutation on the C allele of *JAK2* ($\chi^2 = 60.38$, $P = 7.8 \times 10^{-15}$) (**Fig. 2c**). To investigate this further, we analyzed the association between the *JAK2* mutational status and the rs12343867 genotype in a total of 333 subjects with MPN. *JAK2*^{V617F}-positive subjects (case population; $n = 213$) were compared to *JAK2*^{V617F}-negative subjects (control population, $n = 120$). Subjects heterozygous for rs12343867 and subjects homozygous for the C allele of rs12343867 were more likely to be *JAK2*^{V617F} positive than homozygotes for the T allele (**Table 1**). This indicates that a specific *JAK2* haplotype confers susceptibility to *JAK2*^{V617F}-positive MPN. To further define the involved haplotype, we carried out the same association analysis with a total of eight SNPs within the *JAK2* locus (**Table 1** and **Supplementary Table 1** online). The association between *JAK2* and MPN (independent of *JAK2* mutational status) was also present when a non-MPN Austrian population ($n = 99$) and the entire MPN ($n = 333$) population were compared (**Table 1**). We observed the association of four out of eight SNPs with *JAK2*^{V617F}-positive MPN (**Fig. 3a**). The four SNPs showing significant association (rs3780367, rs10974944, rs12343867, rs1159782) were in linkage disequilibrium (LD) in our MPN cohort (**Fig. 3b**) and two frequent haplotypes (TCTT and GGCC) were predicted with frequencies of 0.50 and 0.45, respectively. No association was observed when *JAK2*^{V617F}-negative subjects were compared with non-MPN controls (data not shown).

To experimentally determine the preferred haplotype that acquired *JAK2*^{V617F}, we took advantage of the frequent occurrence of acquired uniparental disomy on chromosome 9p (9pUPD) in MPN that occurs in clonal myeloid cells carrying *JAK2*^{V617F} (ref. 2). Uniparental disomy on chromosome 9p causes the transition from heterozygosity for *JAK2*^{V617F} to homozygosity for the mutation and a simultaneous loss of the wild-type *JAK2* allele (**Fig. 4a**). Thus, individuals with 9pUPD are always homozygous for *JAK2*^{V617F} and carry only the single *JAK2* haplotype that acquired the mutation. Genotyping of the eight SNPs in 17 subjects with 9pUPD revealed the haplotypes that acquired *JAK2*^{V617F} (**Fig. 4b**). The GGCC haplotype acquired *JAK2*^{V617F} in 88% of all cases analyzed. When we carried out haplotype association in subjects with MPN, the GGCC haplotype was more frequent in *JAK2*^{V617F}-positive subjects than in *JAK2*^{V617F}-negative subjects (haplotype-specific $\chi^2 = 16.87$, $P = 4 \times 10^{-5}$). Several *JAK2* haplotypes were previously reported to be more frequent in individuals with polycythemia vera compared to the other two MPN entities[17]. However, it is unclear how these previously reported *JAK2* haplotypes overlap with the GGCC *JAK2* haplotype described in our study.

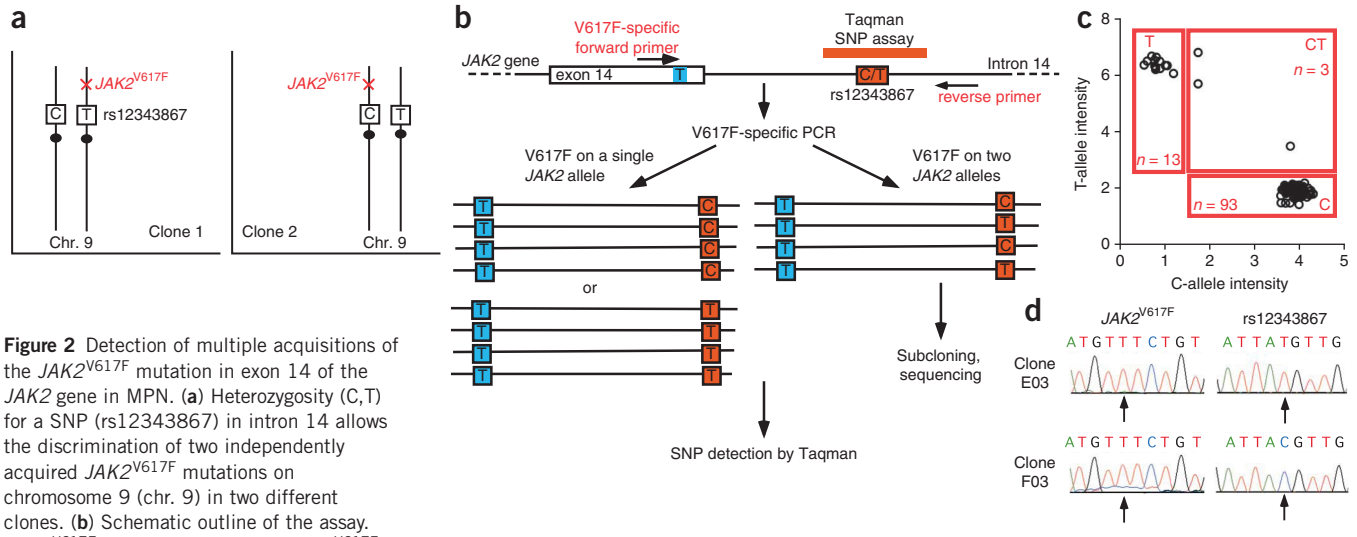

Figure 2 Detection of multiple acquisitions of the *JAK2*^{V617F} mutation in exon 14 of the *JAK2* gene in MPN. (**a**) Heterozygosity (C,T) for a SNP (rs12343867) in intron 14 allows the discrimination of two independently acquired *JAK2*^{V617F} mutations on chromosome 9 (chr. 9) in two different 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 subjects (open circles) by Taqman genotyping. Relative fluorescence of probes specific for the C or T alleles are shown on the *x* and *y* axis. The number (*n*) of individual genotype calls (TT, CT or CC) are shown in red boxes. Subjects with CT genotype calls acquired *JAK2*^{V617F} independently on two *JAK2* alleles. (**d**) Sequence analysis of subcloned PCR products from one of the subjects that acquired *JAK2*^{V617F} on both *JAK2* gene alleles. Two clones (E03 and F03) with different genotypes for the rs12343867 SNP (arrow on right) are shown to carry the *JAK2*^{V617F} oncogenic mutation (arrow on left).

We have identified a common *JAK2* haplotype that preferentially acquires a specific oncogenic mutation and shows that over 80% of all the *JAK2*^{V617F} mutations in MPN occur on this specific haplotype. At present, it is unclear why this association occurs, although there are several potential mechanisms. It is possible that a favorable '*cis* regulatory environment' is present on the GGCC haplotype and that the *JAK2*^{V617F} mutation must occur on this haplotype to induce clonal expansion and MPN development. The nature of this hypothetical *cis* regulatory environment remains unclear. A common hypothesis is that haplotypes contribute to disease by the alteration of gene expression or by a specific modification of protein function through nonsynonymous SNPs. The GGCC haplotype does not contain the promoter region of *JAK2* and the two genotyped SNPs in this region are not in LD with the haplotype. Although it is unlikely that the GGCC haplotype affects *JAK2* gene expression, its influence on *JAK2* mRNA level cannot be ruled out. On the basis of searches in public databases, the SNPs within the GGCC haplotype are not in LD with any nonsynonymous SNPs that might influence protein function. Because MPN is not confined to subjects carrying the GGCC haplotype, it is unlikely that MPN development is restricted to cases where the *JAK2*^{V617F} mutation is acquired in a certain sequence context. In two of three cases with multiple *JAK2*^{V617F} acquisition, two clones coexisted side by side, one with *JAK2*^{V617F} on the TCTT haplotype and the other with it on the GGCC haplotype, respectively (**Fig. 2c**). The TCTT clone predominated in both subjects. This suggests that the GGCC haplotype is unlikely to carry specific properties that are required by the *JAK2*^{V617F} mutation to gain proliferative advantage. It remains to be seen whether the GGCC haplotype bears features that may confer increased mutagenesis and whether exon 12 mutations of *JAK2* are also preferentially acquired on this haplotype.

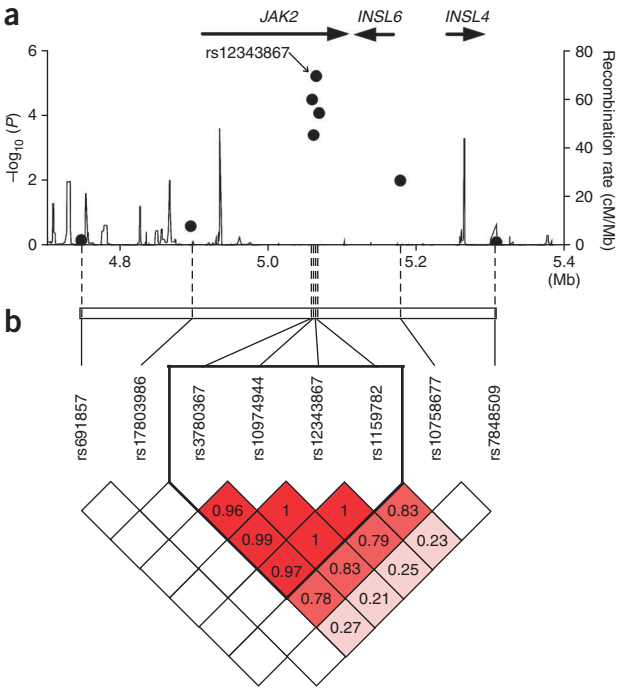

Figure 3 Association analysis results for eight SNPs from the *JAK2* genomic region. (**a**) Association between SNP genotypes and MPN positive and negative for the *JAK2*^{V617F} mutation was analyzed. χ^2-test *P* values are shown on the left axis for each SNP (black dots). Chromosomal positions are based on NCBI build 36 (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 subjects with MPN. Red boxes indicate LD (*D* values shown for SNP pairs).

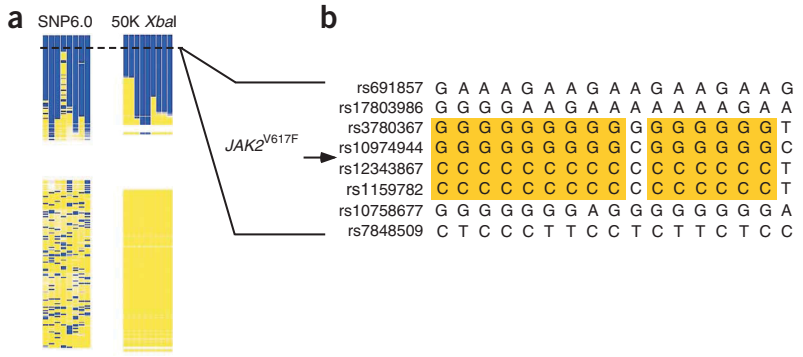

Figure 4 Determination of the *JAK2* gene haplotypes that carry *JAK2*^{V617F} in 17 subjects with uniparental disomy of chromosome 9p (9pUPD). (**a**) Subjects with 9pUPD were identified by loss-of-heterozygosity analysis using either the SNP6.0 or the 50K *Xba*I Affymetrix microarrays. LOH (blue) was called using the dChip program. All subjects had LOH in the *JAK2* locus and were confirmed to be homozygous for the *JAK2*^{V617F} mutation. (**b**) Subjects with 9pUPD carry only the haplotype that acquired *JAK2*^{V617F}. Each subject (columns) was genotyped for eight SNPs (rows). The most common haplotype (GGCC) that carries the oncogenic mutation *JAK2*^{V617F} is highlighted in color. Arrow indicates the position of the *JAK2*^{V617F} mutation relative to the analyzed SNPs.

According to two previous studies, DNA sequence variations in the *APC* and *TP53* genes predispose these loci to somatic mutagenesis[18,19]. One interpretation of our data is that DNA sequence variants can define somatic mutability and could make some haplotypes more susceptible to DNA damage or replication infidelity. A difference in mutability between two haplotypes can explain why *JAK2*^{V617F} preferentially occurs on the GGCC haplotype. If other loci throughout the genome show similar properties as the GGCC *JAK2* haplotype described here, defining disease susceptibility loci using whole-genome association studies may result in the discovery of new somatic mutations in genes within disease susceptibility regions. This is especially true for cancer susceptibilities, as somatic mutations are the underlying cause of cancers.

METHODS

Subjects, DNA samples, genotyping. Collection of blood samples was done at the Medical University of Vienna and was approved by the local ethics committee. Written informed consent was obtained from participating subjects in accordance with the Declaration of Helsinki. The diagnosis on MPN was established by the WHO criteria. Peripheral blood samples were fractionated to isolate mononuclear cells and granulocytes using density gradient centrifugation. Granulocyte DNA was used for determination of *JAK2*^{V617F} mutational status and for analysis of uniparental disomy. For SNP genotyping, we used DNA from nonmyeloid cells to eliminate false homozygosity calls due to the presence of uniparental disomy on chromosome 9p, which is often present in DNA samples derived from myeloid cells of subjects with MPN[2]. The *JAK2*^{V617F} mutational status of subjects was determined by PCR as previously described[9]. SNP genotyping was done using commercially available Taqman assays (Applied Biosystems) analyzed on the 7900HT real-time PCR instrument using standard protocols. The following assays were used in this study: C____730925, C__34291999, C__27515396, C__31941696, C__31941689, C___1417119, C___1417053 and C__30593278.

Table 1 Genotype-specific association of SNPs from the *JAK2* locus on chromosome 9 and MPN

Case population	Control population	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]χ^2 test. [b]Cochran-Armitage trend test.

Culture and genotyping of hematopoietic progenitors. Peripheral blood mononuclear cells were isolated using standard density gradient centrifugation. Cells were plated in the H4431 methylcellulose media (StemCell Technologies) at 3×10^5 cells/ml according to the manufacturer's recommendations. Day 14 colony forming units (CFU) representing erythroid and granulocytic-monocytic progenitor clones were individually picked into 5% of Chelex-100 resin solution (Biorad) in 5 mM Tris pH 8, 0.5% Triton X-100. The lysates were incubated for 15 min at 56 °C and boiled for an additional 8 min. We used 1 µl of lysate for PCR-based genotyping. Deletions were detected by loss of heterozygosity analysis using microsatellite PCR for *D20S96* (del20q) and *D13S153* (del13q). The *JAK2*^{V617F} mutational status of CFUs was determined by quantitative allele-specific PCR as previously described.

Microarray analysis. To detect loss of heterozygosity in DNA samples, we used the GeneChip SNP 6.0, the 50K *Xba*I, and 10K *Xba*I Mapping arrays (Affymetrix). Data analysis was done with the Genotyping Console version 2.1 (Affymetrix) and the dChip[20] software packages.

Analysis of multiple occurrence of *JAK2*^{V617F}. The analysis consisted of two steps. Subjects heterozygous for rs12343867 and also positive for *JAK2*^{V617F} in granulocyte genomic DNA were used for the assay. Allele-specific PCR was done with primers V617F-F and intr14-R (**Supplementary Table 2** online) using AmpliTaq Gold polymerase in Gold buffer with 1.5 mM magnesium chloride (Applied Biosystems). Thirty cycles were done in 2720 Thermal Cyclers (Applied Biosystems) using 95 °C initial denaturation (10 min), followed by cycles of 94 °C 30 s, 56 °C 30 s, and 72 °C 30 s and a 10 min final extension at 72 °C. In the second step, 5–10 ng of PCR product was used for Taqman assays to determine the *JAK2* allele that acquired the *JAK2*^{V617F} mutation. This was done using the commercial Taqman assay C__31941689 and the 7900HT Thermal Cycler (Applied Biosystems).

Statistical analysis. Statistical analysis of SNP association was done using PLINK software[21]. We carried out allelic and genotypic association tests for eight SNPs in PLINK (χ^2 test, Fisher's exact test, Cochran-Armitage trend test) comparing different case-control groups. The corresponding unadjusted *P* values shown in **Table 1** remained significant after correction for multiple testing using Bonferroni, Holm, Sidak and FDR control methods. Haplotype phases (computed using expectation-maximization algorithm) and haplotype associations were both determined in PLINK. PLINK-derived linkage disequilibrium (LD) values were subsequently visualized using Haploview software[22]. We carried out a χ^2 test for the distribution of rs12343867 alleles acquiring *JAK2*^{V617F} mutation in heterozygotes using the R statistical package. We calculated odds ratios for SNP genotype associations using the SNPStats statistical web tool.

URLs. Microarray analysis software dChip 2008, http://www.hsph.harvard.edu/~cli/complab/dchip; PLINK version 1.04, http://pngu.mgh.harvard.edu/purcell/plink; HapMap recombination rates, http://ftp.hapmap.org/recombination/2006-10_rel21_phaseI+II/rates; R statistical package (version 2.8.0), http://www.R-project.org; Haploview version 4.1, http://www.broad.mit.edu/mpg/haploview; SNPStats, http://bioinfo.iconcologia.net/SNPStats.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

R.K. designed the study and drafted the paper with assistance of D.O. and A.H.; D.O., T.B. and R.J. performed the experiments; D.O. and A.H. performed statistical analyses; T.B., B.G., H.G. and I.P. coordinated and performed the case and control sample collection and clinical management of cases.

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3.5 Manuscript # 3: The role of *JAK2* GGCC haplotype and *TET2* gene in familial myeloproliferative neoplasms

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After the identification of *JAK2* 'GGCC' haplotype as a major predisposition factor in myeloproliferative neoplasms (Jones et al, 2009; Kilpivaara et al, 2009; Olcaydu et al, 2009a; Olcaydu et al, 2009b) it was reasonable to consider that it can play an important role in familial clustering of MPN. *TET2* (Tefferi et al, 2009) and *CBL* (Grand et al, 2009) have been discovered to be frequently somatically mutated in MPN. The roles of these mutations in familial MPN were not investigated before.

We investigated the frequency of germline and somatic mutations in *TET2*, *CBL* and *MPL* genes in 88 MPN cases from 52 families. The entire *TET2* gene, exons 8 and 9 of *CBL*, and exon 10 of *MPL* were sequenced. No *CBL* or *MPL* mutations were found in familial MPN cases. The sequencing of *TET2* gene yielded 7 somatic and 5 germline mutations. However, all of the germline mutations either were common variants found in the public databases or did not segregate with the disease in the family. Thus, there is no evidence that germline mutations in any of those three genes play a role in familial MPN (Olcaydu et al, 2011).

We also genotyped the familial MPN cases for two SNPs (rs10974944 and rs12343867) tagging *JAK2* 'GGCC' haplotype and compared the frequencies with 684 population-matched sporadic MPN and 203 healthy controls. Data analysis showed that 'GGCC' haplotype predisposed to the development of JAK2-positive familial MPN and has no effect on JAK2-negative familial MPN. Moreover, there was no significant difference in 'GGCC' haplotype frequency distribution between sporadic and familial MPN cases. The penetrance of familial MPN was estimated to be 31-35%, which is several orders of magnitude higher than for 'GGCC' haplotype (0.02%). Thus, 'GGCC' haplotype by itself is not responsible for familial clustering of MPN, but has an additive effect on familial susceptibility (Olcaydu et al, 2011).

In this paper I have participated in the genotyping for 'GGCC' haplotype and subsequent statistical analysis of the SNP genotyping data.

The role of the *JAK2* GGCC haplotype and the *TET2* gene in familial myeloproliferative neoplasms

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

Myeloproliferative neoplasms constitute a group of diverse chronic myeloid malignancies that share pathogenic features such as acquired mutations in the *JAK2*, *TET2*, *CBL* and *MPL* genes. There are recent reports that a *JAK2* gene haplotype (GGCC or 46/1) confers susceptibility to *JAK2* mutation-positive myeloproliferative neoplasms. The aim of this study was to examine the role of the *JAK2* GGCC haplotype and germline mutations of *TET2*, *CBL* and *MPL* in familial myeloproliferative neoplasms.

Design and Methods

We investigated patients with familial (n=88) or sporadic (n=684) myeloproliferative neoplasms, and a control population (n=203) from the same demographic area in Italy. Association analysis was performed using tagged single nucleotide polymorphisms (*rs10974944* and *rs12343867*) of the *JAK2* haplotype. Sequence analysis of *TET2*, *CBL* and *MPL* was conducted in the 88 patients with familial myeloproliferative neoplasms.

Results

Association analysis revealed no difference in haplotype frequency between familial and sporadic cases of myeloproliferative neoplasms (P=0.6529). No germline mutations in *TET2*, *CBL* or *MPL* that segregate with the disease phenotype were identified. As we observed variability in somatic mutations in the affected members of a pedigree with myeloproliferative neoplasms, we postulated that somatic mutagenesis is increased in familial myeloproliferative neoplasms. Accordingly, we compared the incidence of malignant disorders between sporadic and familial patients. Although the overall incidence of malignant disorders did not differ significantly between cases of familial and sporadic myeloproliferative neoplasms, malignancies were more frequent in patients with familial disease aged between 50 to 70 years (P=0.0198) than in patients in the same age range with sporadic myeloproliferative neoplasms.

Conclusions

We conclude that the *JAK2* GGCC haplotype and germline mutations of *TET2*, *CBL* or *MPL* do not explain familial clustering of myeloproliferative neoplasms. As we observed an increased frequency of malignant disorders in patients with familial myeloproliferative neoplasms, we hypothesize that the germline genetic lesions that underlie familial clustering of myeloproliferative neoplasms predispose to somatic mutagenesis that is not restricted to myeloid hematopoietic cells but cause an increase in overall carcinogenesis.

Key words: *JAK2*-V617F, 46/1 haplotype, germline, predisposition, somatic mutagenesis.

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Introduction

Myeloproliferative neoplasms (MPN) constitute a group of phenotypically diverse chronic myeloid malignancies that are characterized by the presence of clonal hematopoiesis and an excessive production of terminally differentiated myeloid blood cells. The so-called "classic Philadelphia-chromosome negative MPN" encompass three distinct diseases, namely polycythemia vera (PV), essential thrombocythemia and primary myelofibrosis. The identification of the V617F mutation of the *JAK2* gene (*JAK2*-V617F) led to an important breakthrough in the understanding of the pathogenesis of MPN.[1-4] Several studies have shown that the somatic acquisition of *JAK2*-V617F establishes constitutive activation of JAK-STAT signaling, erythropoietin-independent growth of erythroid progenitor cells and the MPN phenotype in murine bone marrow transplant models.[3,5,6] The *JAK2*-V617F mutation is present in the majority of patients with PV (90-98%), whereas only about 50% of patients with essential thrombocythemia and primary myelofibrosis are affected.[7] However, further investigations have revealed that other oncogene mutations that alter JAK-STAT signaling occur frequently in patients negative for the *JAK2*-V617F mutation, emphasizing the role of this signaling pathway in the pathogenesis of MPN. Mutations and deletions in exon 12 of the *JAK2* gene (*JAK2*-ex12) contribute to the pathogenesis of PV and occur in about 20% of *JAK2*-V617F-negative PV patients, whereas mutations in the thrombopoietin receptor gene *MPL* have been identified in 1-5% of cases of essential thrombocythemia and primary myelofibrosis.[8,9]

Recent investigations have shown that somatic acquisition of genetic aberrations is not the only pathogenic mechanism, but that inherited genetic factors also play a pivotal role in the development of MPN. Independent studies have identified a *JAK2* haplotype that has an increased risk of acquiring mutations at this locus. Several studies showed a non-random distribution of the *JAK2*-V617F mutation between the two parental *JAK2* alleles and that more than 80% of *JAK2*-V617F mutations were acquired on this particular *JAK2* gene haplotype, which is referred to as the GGCC or 46/1 haplotype.[10-12] Subsequent association analysis revealed that *JAK2*-ex12 mutations also occur more frequently on the *JAK2* GGCC haplotype.[13] Thus, this sequence variant of the *JAK2* gene confers susceptibility to *JAK2* mutation-positive MPN.

Recent data indicate that the GGCC haplotype predisposes to *MPL* mutation-positive MPN, although the reported association is significantly weaker than that between the GGCC haplotype and *JAK2* mutations.[14] The mechanisms by which this observed difference in mutability between haplotypes is accomplished remains to be elucidated. As the *JAK2* GGCC haplotype has not been evaluated in a large series of familial MPN, we investigated a demographically matched population of patients with familial and sporadic MPN. With its germline predisposition to acquire *JAK2* mutations, the GGCC haplotype has been hypothesized to explain familial clustering of MPN.[11,12] Although MPN occurs sporadically in most cases, familial clustering has been reported.[15-17] Common mutations involved in the pathogenesis of MPN, such as *JAK2*, *MPL* and *TET2* mutations are not inherited, but somatically acquired also in familial cases.[16,18,19] One exception is the *MPL*-S505N mutation that was found to be a germline mutation in a Japanese pedigree with hereditary thrombocythemia.[20] An inherited mutation of the *TET2* gene causing a frame shift and premature stop was recently identified in a patient with PV.[21] Furthermore, germline *CBL* mutations were reported in patients with juvenile myelomonocytic leukemia,[22] but their role in familial MPN remains to be elucidated. The aim of this study was to investigate the pathogenic relevance of mutations of *JAK2*, *TET2*, *CBL* and *MPL* genes in familial clustering of MPN.

Design and Methods

Patients, blood sampling and DNA isolation

A total of 982 consecutive patients with apparently sporadic MPN, who were diagnosed and followed from 1973 to 2010 at the Department of Hematology, Fondazione IRCCS Policlinico San Matteo of Pavia, were interviewed to determine whether they actually had a family hisory of MPN. Of these 982 patients, DNA for molecular evaluation was available for 772 (79%), who were, therefore, included in this study. Patients were defined as familial cases if two or more individuals within the same pedigree were affected. Some of these patients were included in a prior clinical study.[17] The diagnosis of MPN was made in accordance with the criteria in use at the time of the first observation of the patients.[23-27] A patient was defined as being in blast phase if his or her bone marrow or peripheral blood blast cell count was 20% or higher, in accordance with the World Health Organization (WHO) 2008 criteria.[23] Patients with acute myeloid leukemia were not included in the analysis of malignancies other than MPN as the blast phase is considered to be a progression from MPN. We considered solid tumors and lymphoproliferative disorders occurring both before and after the diagnosis of MPN as associated malignancies. The diagnosis of solid tumors was based on biopsies of the suspected lesion. The diagnosis of lymphoproliferative disorders was made according to the WHO 2008 criteria. Subjects with a normal hemogram (n=43) or a hematologic reactive condition (n=160) were used as a demographically matched control population (n=203). Of the latter, 34 patients were diagnosed as having reactive leukocytosis and 126 as having reactive erythrocytosis. Peripheral blood from patients was sampled after written informed consent had been obtained. Blood sampling, cell fractionation and DNA isolation from granulocytes and CD3-positive T lymphocytes were conducted as previously described.[28] This study was approved by the institutional ethics committee (Comitato di Bioetica, Fondazione IRCCS Policlinico San Matteo) and the procedures were conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000.

JAK2 mutation analysis, single nucleotide polymorphism genotyping and sequence analysis

Molecular studies were performed in all patients with familial (n=88) and sporadic (n=684) MPN for whom DNA was available. The *JAK2*-V617F mutation burden was assessed using a quantitative polymerase chain reaction (qPCR)-based allelic discrimination assay, as previously described with the following modifications.[29] All reactions were carried out on a RotorGene 6000TM real-time analyzer on a 100-well Gene Disk (Corbett Life Sciences, Mortlake, NSW, Australia) in a final volume of 12 μL containing 1x Brilliant SYBR Green QPCR master mix (Stratagene, Cedar Creek, TX, USA) 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 previously described.[30] Two tagging single nucleotide polymorphisms (SNP) 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) according to standard protocols and data were analyzed on a 7900HT real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). Cytogenetic aberrations in three patients of the same pedigree with familial MPN were identified by high-resolution SNP genotyping using GeneChip SNP 6.0 Mapping arrays and data were analyzed with Genotyping Console software version 3.0.2 (Affymetrix, Santa Clara, CA, USA).

Sequences of *TET2*, *CBL* and *MPL* genes were analyzed in patients with familial MPN for whom sufficient amounts of DNA were available (n=88). Nine coding exons of the *TET2* gene, exons 8 and 9 of *CBL* (carrying most *CBL* mutations reported so far[31,32]), 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. Data were analyzed using the Sequencher Software (version 4.9; Gene Codes, Ann Arbor, MI, USA). The sequences of primers and conditions used for PCR amplification and sequence analysis are summarized in *Online Supplementary Table S1*.

A detailed description of the statistical methods used in this study is available in the *Online Supplementary Methods*.

Results

Patients' characteristics

After interview-based investigation of a family history of MPN in 772 patients with apparently sporadic MPN from the same demographic area in Italy, 88 (11%) were considered familial cases (52 families) and 684 (89%) as sporadic cases. Of the 52 Italian familial MPN pedigrees, DNA for molecular analysis was available from one affected member of 18 families, two affected members of 32 families and three affected members of two families. Molecular assessment was performed in 320 (41%) of the 772 patients at diagnosis (29 familial and 291 sporadic cases) and in 452 patients (59%) during follow-up (59 familial and 393 sporadic cases). In the latter, the median duration of MPN before molecular analysis was 6.1 years (range, 0.3-22 years) in familial cases and 4.3 years (range, 0.1-25.1 years) in sporadic cases. The patients' demographic and clinical characteristics at diagnosis are summarized in *Online Supplementary Table S2*.

The JAK2 GGCC haplotype in familial and sporadic myeloproliferative neoplasms

In order to determine the role of the *JAK2* GGCC haplotype in familial MPN, sporadic MPN and a non-MPN control population, the allele frequencies of a tagging SNP of the GGCC haplotype (*rs10974944*) were compared in the cohorts of patients. The G variant of *rs10974944*, resembling the high-risk variant for the acquisition of *JAK2* mutations, was more frequent in familial and sporadic MPN (0.44 and 0.42, respectively) than in the control population (0.27). Assuming that the GGCC haplotype is causative in familial clustering of MPN, we expected a higher frequency of the risk variant in familial MPN compared to sporadic cases. There was, however, no significant difference in the allele frequencies of the G variant of *rs10974944* between familial and sporadic cases of MPN (P=0.5851). Furthermore, association analysis applying a co-dominant genotypic model revealed a significant association between heterozygosity and homozygosity for the G variant of *rs10974944* and the presence of the *JAK2*-V617F mutation in both familial and sporadic cases of MPN, when compared to the control population (Table 1). Association analysis comparing only *JAK2*-V617F-positive familial and sporadic cases of MPN with the control population revealed a significant association between the risk haplotype and the *JAK2* mutation, whereas the comparison of *JAK2*-V617F-negative MPN with the control population did not show a significant correlation, either in familial cases or in sporadic ones.

To test the hypothesis that the *JAK2* GGCC haplotype plays a role in *JAK2*-V617F-positive familial MPN, we compared the genotype frequencies of *rs10974944* in

Table 1. Genotype association analysis of *rs10974944* (tagging SNP of the *JAK2* GGCC haplotype) and *JAK2*-V617F.

Case population	Control population	Genotype frequency (%) case population			Genotype frequency (%) control population			Odds ratio (95% CI)			
		CC	GC	GG	CC	GC	GG	CC	GC	GG	*P* value
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

CI, confidence interval; V617F+, JAK2-*V617F-positive MPN; V617F-*, JAK2-*V617F-negative MPN.*

JAK2-V617F-positive familial MPN and *JAK2*-V617F-positive sporadic MPN. Genotypic association analysis did not show a significant correlation between the GGCC haplotype and familial clustering in MPN (P=0.6529, Table 1). Thus, the risk of acquiring *JAK2* mutations conferred by the GGCC haplotype does not differ between cases of sporadic and familial MPN regardless of the *JAK2* mutation status. In conclusion, the *JAK2* GGCC haplotype predisposes to the acquisition of *JAK2* mutations also in familial MPN, but does not underlie familial clustering.

Sequence analysis of TET2, CBL *and* MPL

Somatic mutations in genes that are relevant to 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 DNA in a total of 88 affected patients with a family history of MPN (52 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 and seven patients carried a missense mutation (Table 2). In three patients we identified a nonsense mutation resulting in a premature stop-codon, one of which has been previously described.[19] To evaluate whether these mutations were somatically acquired or inherited, we subsequently performed sequence analysis of DNA samples from T lymphocytes of these patients. In five cases we could confirm the presence of the same mutation in granulocyte DNA as well as T lymphocyte DNA, whereas seven mutations were only detectable in the granulocyte sample and were, therefore, somatically acquired. We identified one patient (MPC08-188) who carried two mutations of the *TET2* gene. 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 of which were previously reported as normal variants (Table 2). Two patients carried a newly identified *TET2* mutation (A241V and R1440Q) that was present in both granulocyte and T lymphocyte DNA. In order to investigate the segregation of these two germline mutations with the MPN phenotype, we investigated whether the other affected member of the pedigree carried the same mutation. For patient MPC08-188, who harbored an A241V germline mutation as well as a somatic R550X mutation of *TET2*, no DNA was available from the other affected family member for molecular analysis. In the case of patient 377, the other family member with apparent MPN did not carry any mutation of the *TET2* gene, thus excluding segregation of the R1440Q mutation with the disease phenotype. Among all of the 52 Italian MPN pedigrees analyzed, 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.

Genetic heterogeneity of somatic mutations and penetrance in familial myeloproliferative neoplasms

We studied an Australian family with three individuals affected by MPN using high-resolution SNP genotyping and tested for *JAK2* and *MPL* mutations (Figure 1A). Of the three affected members, one who was diagnosed with essential thrombocythemia was positive for the *MPL*-W515L mutation (MPD214) and one with a diagnosis of primary myelofibrosis had the *JAK2*-V617F mutation as well as a 5.7 mega base pair deletion on chromosome 22q (MPD227). The third evaluated member of this family was diagnosed with primary myelofibrosis and was negative for *MPL* and *JAK2* mutations, but carried deletions on chromosomes 2p, 7q and 15q (MPD219) (Figure 1A). All of these molecular defects were somatic (*data not shown*). Notably, the patient who carried the *JAK2*-V617F mutation was a heterozygous carrier of the *JAK2* GGCC haplotype, whereas the other two affected members were both homozygous for the non-risk haplotype (Figure 1A). Thus, there is no segregation of the *JAK2* GGCC haplotype with the MPN phenotype in this family. Of all patients with familial MPN analyzed in this study, 24 (27.3%) did not harbor the GGCC haplotype, 51 (58.0%) were heterozygous and 13 (14.8%) were homozygous carriers of the *JAK2* gene risk variant (Figure 1B, Table 1). These data demonstrate that up to one third of patients with familial MPN develop the disease although they do not carry the

Table 2. Summary of *TET2* mutations in patients with familial MPN (n=88).

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	(41, 42)
F17P1	PV	11	G/T	+	+	V1718L	VAR_058190	(41, 43)
F20P2	PV	11	C/T	+	+	P1723S	VAR_058192	(41, 42)
MPC08-188	Post-ET MF	3	C/T	+	-	R550X	NA	(19)
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

ins: insertion; del: deletion; NA: not available; ET: essential thrombocythemia; MF: myelofibrosis; PMF: primary myelofibrosis; PV: polycythemia vera.

JAK2 risk haplotype.

In order to further characterize the contribution of the *JAK2* GGCC haplotype to the familial occurrence of MPN, we performed penetrance estimation calculations. The penetrance in familial MPN was estimated to be 31-35%, a range caused by variable inclusion of some MPN family members. In comparison, the penetrance of the *JAK2* GGCC haplotype was lower by approximately three orders of magnitude (0.02%). This difference in estimated penetrance provides further evidence that the familial predisposition to develop MPN is much stronger than the one conferred by the *JAK2* GGCC haplotype.

Malignancies in patients with familial and sporadic myeloproliferative neoplasms

The pedigree shown in Figure 1A provides evidence that the MPN predisposition is not restricted to somatic mutations of *JAK2* only. We hypothesize that individuals with a family history of MPN carry a germline genetic defect that confers a predisposition to increased somatic mutagenesis and results in a higher incidence of malignant disorders. To investigate this hypothesis, we compared the occurrence of malignancies (solid tumors and hematologic malignancies) in familial and sporadic cases of MPN and evaluated their association with the *JAK2* GGCC haplotype. In the entire cohort of 772 MPN patients, 52 (6.7%) malignancies were recorded, of which 21 (40%) occurred before and 31 (60%) after the diagnosis of MPN, as reported in *Online Supplementary Table S3*. Among 88 patients with a family history of MPN, ten (11.4%) were diagnosed with a malignancy, whereas 42 (6.1%) of 684 patients with sporadic MPN had a malignancy other than MPN (Fisher's exact test P=0.0721). Figure 2 shows the frequencies of all observed malignancies in the familial and sporadic cases of MPN.

Two different statistical analyses were carried out in order to investigate the occurrence of malignancies in familial and sporadic MPN. In the first setting, we used a case-control model to assess the risk of developing malignancies throughout the patients' lives (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 did sporadic cases [odds' ratio (OR) 1.96, 95%

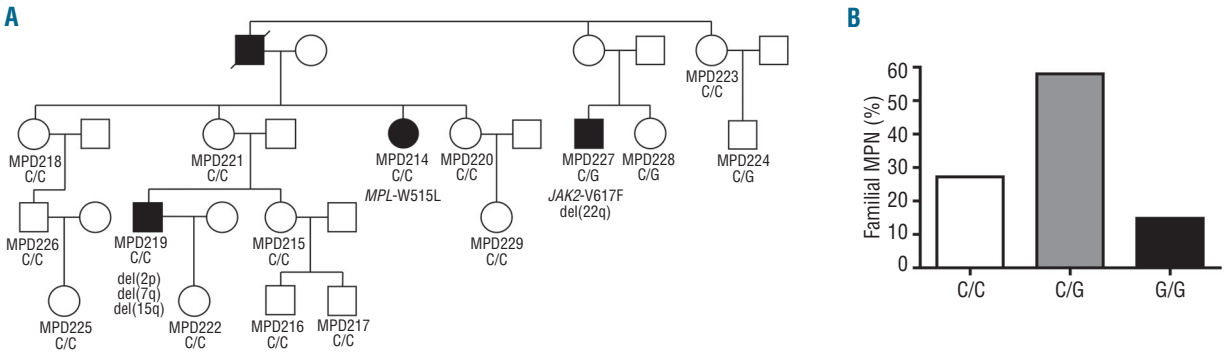

Figure 1. The *rs10974944* genotype and genetic heterogeneity in familial MPN. (A) Pedigree showing a MPN family with three affected members carrying distinct genetic aberrations. Family members with manifest MPN are shown as filled symbols with females represented as circles and males as squares. Patients who 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 patient with familial MPN diagnosed with primary myelofibrosis was heterozygous for *rs10974944* (MPD227), positive for the *JAK2*-V617F mutation and had a deletion on the long arm of chromosome 22 [del(22q)]. One of the other affected members was diagnosed with essential thrombocythemia and carried the *MPL*-W515L mutation (MPD214), whereas the other affected individual (MPD219), diagnosed with primary myelofribrosis, 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.

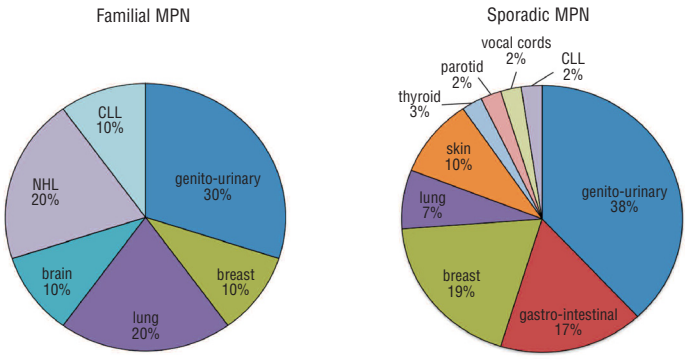

Figure 2. Malignancies in patients with familial or sporadic MPN. Pie charts represent the frequencies of malignant disorders in patients with familial or sporadic MPN. Type of cancer and frequencies in percentages are given for both groups. Lung and brain cancer as well as non-Hodgkin's lymphomas (NHL) and chronic lymphocytic leukemia (CLL) were more frequent in patients with familial MPN than in those with sporadic MPN. On the other hand, gastro-intestinal tumors, breast cancer and tumors of the skin were more frequent in patients with sporadic MPN.

confidence interval (95% CI): 0.84-4.16; *P*=0.0657]. After adjustment for age at diagnosis of MPN, by using four age categories (< 40 years, 40-55 years, 55-65 years, >65 years) with similar numbers of patients in each group (*Online Supplementary Figure S1*), malignancies remained more frequent in familial cases of 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 excluding an influence of the *JAK2* haplotype on the risk of developing other malignancies in patients with familial MPN.

Next, the same analysis was conducted considering the patients' age at last follow-up (median 58 years; range, 18.7-92.2 years). Patients were grouped into three age categories (<50 years, 50-70 years, >70 years) with approximately equal numbers of subjects in each group and the frequency of malignancies was compared between the age categories in sporadic and familial MPN (Figure 3). Although the difference was not statistically significant, overall malignancies were more frequent in familial than in sporadic MPN (OR 1.77, 95% CI: 0.84-3.71; *P*=0.1174). Restricting the analysis to patients in the middle age group (50-70 years), the estimated risk of developing malignancies other than MPN was higher among the patients with familial MPN (OR 3.13, 95% CI: 1.13-8.66; *P*=0.0198).

In a second statistical model, we compared the incidence of malignancies after the diagnosis of MPN between familial and sporadic cases. The incidence of malignancies was 11.7 per 1000 person-years in familial cases and 6.0 per 1000 person-years in sporadic MPN, with an incidence rate ratio of 1.95 (*P*=0.11). After adjustment for age at MPN diagnosis the incidence rate ratio remained similar (1.93), thus excluding an effect of age at diagnosis on the incidence of malignancies other than MPN. Figure 4 illustrates the comparative Kaplan-Meier estimates of cancer-free survival in both groups of patients. Subjects with familial MPN seemed to have poorer cancer-free survival when compared to patients with sporadic MPN (log-rank test, *Z*=1.4776; *P*=0.1395). The observed difference in cancer-free survival was not, however, statistically significant, probably due to the overall limited number of study subjects with a family history of MPN and the low number of malignancies other than MPN in both populations of patient (7 in 88 familial cases of MPN and 24 in 684 cases of sporadic MPN). Thus, larger cohorts of patients are necessary in order to confirm the results of this study. Nevertheless, our data suggest that patients with familial clustering of MPN might harbor a yet unidentified predisposition to develop malignant disorders in general, independently of the *JAK2* haplotype.

Discussion

The identification of a common genetic variation that influences mutability at the *JAK2* gene locus raised the hypothesis that this inherited disease predisposition might underlie familial clustering of MPN. In order to determine the role of the GGCC haplotype in familial occurrence of MPN, we investigated the *JAK2* gene haplotype in the currently largest series of patients with a family history of MPN. Hypothesizing that the *JAK2* GGCC haplotype explains familial clustering, we expected to observe a higher frequency of the haplotype in familial cases than in sporadic ones. Our data demonstrate that although the GGCC haplotype is more frequent in familial MPN than in the control population, the haplotype frequency and the risk of acquiring a mutation in the *JAK2* gene do not differ between familial and sporadic cases. Accordingly, association analysis did not reveal a significant correlation between the *JAK2* GGCC haplotype and the acquisition of *JAK2*-V617F when familial and sporadic cases were compared. About 30% of patients with familial MPN do not carry the *JAK2* GGCC haplotype. Furthermore, penetrance in familial MPN is about three orders of magnitude

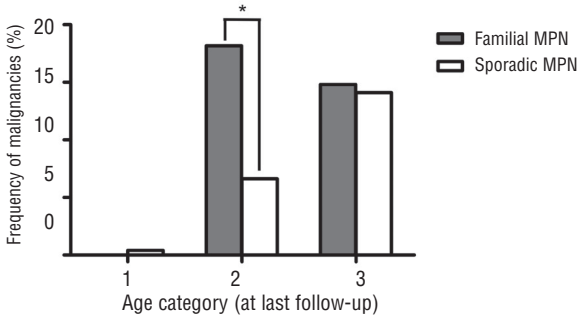

Figure 3. Frequency of malignancies in patients with familial and sporadic MPN according to age at last follow-up. Patients were grouped into three age categories (<50 years, 50-70 years, >70 years) according to age at last follow-up. Gray bars represent familial and white bars sporadic MPN cases. There was only one patient with sporadic MPN 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 higher in patients with familial MPN than in those with sporadic MPN (*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).

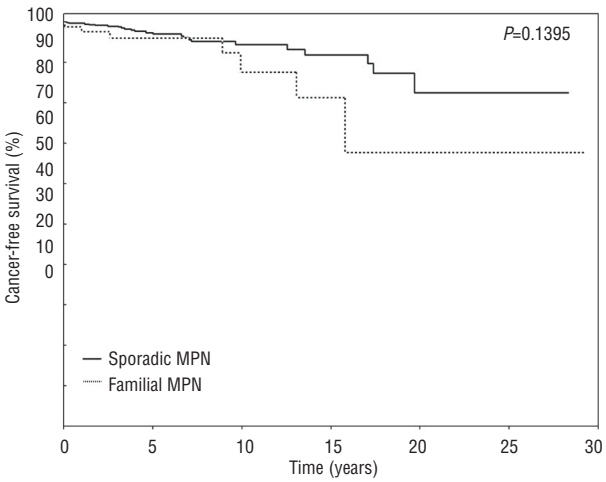

Figure 4. Cancer-free survival after the diagnosis of MPN in patients with familial or sporadic MPN. Kaplan-Meier estimation of cancer-free survival in familial MPN (dashed line) and sporadic MPN (black line). The x-axis shows time after MPN diagnosis in years and the y-axis represents the percentage of patients without malignancy. Patients with a familial history of MPN had a slightly lower cancer-free survival than sporadic MPN cases. However, the difference in cancer-free survival was not statistically significant (*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 patients with sporadic MPN).

higher than penetrance of the *JAK2* GGCC haplotype. These data are consistent with previous reports that excluded linkage in the *JAK2* gene locus using microsatellite marker analysis in four pedigrees with familial PV.[15] Taken together, these observations further support the hypothesis that the GGCC haplotype confers susceptibility to *JAK2* mutation-positive MPN, but does not explain familial clustering.

Acquired mutations in genes relevant to the pathogenesis of MPN have been postulated to be candidates for inherited predispositions to familial MPN. Mutations of the thrombopoietin receptor gene *MPL* have been identified in familial as well as sporadic cases of essential thrombocythemia.[20,33] Only one case of a germline *TET2* mutation in familial MPN has been described so far.[21] There is a recent report of inherited mutations of the *CBL* gene in juvenile myelomonocytic leukemia.[22] In order to gain further insights into the role of *TET2*, *CBL* and *MPL* mutations in familial clustering of MPN, we analyzed the sequence of these genes in a unique cohort of 88 patients with familial MPN. In summary, we could not identify mutations of *TET2*, *CBL* or *MPL* that are inherited and segregate with the disease in our cohort of patients with familial MPN. Hence, the germline genetic defect underlying familial clustering of MPN remains to be identified.

Previous studies in familial MPN found an earlier onset of disease and significant telomere shortening in second-generation MPN patients, providing evidence for disease anticipation in familial MPN.[34] A large-scale study including more than 24,000 first-degree relatives of about 11,000 patients with MPN revealed that the risk of developing MPN was higher in relatives of MPN patients.[35] Furthermore, the authors reported an increased risk of hematologic malignancies (chronic myeloid leukemia and chronic lymphocytic leukemia) and solid tumors (malignant melanoma and brain cancer) among relatives of patients with MPN.[35] Together with the results of our study, these data support the hypothesis that there is a general inherited predisposition to familial MPN that precedes the acquisition of "phenotype-initiating" mutations such as *JAK2*-V617F and *MPL*-W515L. Unlike the *JAK2* GGCC haplotype, this germline predisposition seems to promote the acquisition of various genetic defects, as shown in Figure 1A. If so, somatic mutability might not only be restricted to MPN but may manifest as an increase in overall carcinogenesis, as suggested by the data of Landgren *et al.*[35]

To investigate the hypothesis that patients with familial MPN have increased somatic mutability, we evaluated the incidence of malignancies in a unique cohort of patients with familial MPN from 52 MPN pedigrees. We noted a difference in the occurrence of malignant disorders between patients with familial or sporadic MPN, particularly in patients aged 50 to 70 years at last follow-up. Statistical analysis revealed that patients with a family history of MPN have an up to 3-fold increased risk of developing other malignancies. Furthermore, we noted a difference in cancer types and frequencies that occur in patients with familial or sporadic MPN. Whereas tumors of the gastro-intestinal tract (17%) and skin (10%) were frequent in patients with sporadic MPN, we did not identify any such case among patients with familial MPN. Conversely, chronic lymphocytic leukemia (10%) and tumors of the brain (10%) were exclusively found in patients with familial MPN and non-Hodgkin's lymphomas were much more frequent in familial cases (20%) than in sporadic ones (2%). Kaplan-Meier estimates of cancer-free survival in patients with familial or sporadic MPN suggested a decreased cancer-free survival in familial cases, although the difference was not statistically significant. This might be due to the fact that despite being one of the largest cohorts of familial MPN studied, the number of subjects was limited and the overall incidence of malignant disorders was low in both groups of patients.

In accordance with previous studies, our data suggest that patients with familial MPN have a higher risk of malignant disorders. The number of patients with familial MPN available for investigation was limited, given that this is a rare disorder. Larger cohorts of patients are needed in order to further characterize inherited disease predispositions and to identify the genetic defect(s) that underlie familial clustering of MPN. Based on the results of this study, we hypothesize that there is a mutation – of, for example, a tumor suppressor gene – which establishes increased somatic mutability and promotes the consecutive acquisition of genetic defects. It remains unclear why a mutation in a general tumor suppressor gene would preferentially produce an MPN phenotype. However, previous reports in literature describe that certain mutations or deletions of tumor suppressor genes, such as *TP53*, *VHL*, *APC*, *BRCA* and *CDKN2A*, exhibit specific genotype-phenotype correlations.[36-40] Previous studies of the *JAK2* GGCC haplotype and the existence of familial MPN demonstrate that germline genetic factors influence somatic mutagenesis and cause various cancer phenotypes. The identification of the mutations that cause familial MPN will further advance our understanding of the interplay between germline and somatic mutagenesis.

Authorship and Disclosures

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3.6 Manuscript # 4: Rare germline variants in regions of loss of heterozygosity may influence clinical course of hematological malignancies

Harutyunyan A. et al.,

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Somatic genetic lesions and germline mutations can interact in the course of pathogenesis of various cancers. There are numerous examples of cancers when one allele is mutated germline while the second allele is affected by somatic point mutation or chromosomal aberration (Comino-Mendez et al, 2011; Lee et al, 1987; Varela et al, 2011).

In the paper we investigated a patient with MPN who had acquired 14q UPD in the course of the disease. We performed exome sequencing on the granulocyte DNA sample on Illumina sequencing platform and analyzed the mutations in the region of acquired uniparental disomy on chromosome 14q. The analysis yielded several germline mutations, among them a nonsense mutation of Fanconi anemia complementation group M gene (*FANCM*), R658X. This truncating mutation had become homozygous as a result of 14q UPD. The clinical course of the disease fitted with the acquisition of the mutation. Initially patient was diagnosed with PV, but after several years, with the acquisition of 14q UPD, the patient developed anemia and later transformed to sAML (Harutyunyan et al, 2011a).

MPN patients frequently carry different large-scale somatic chromosomal aberrations which may accidentally interact with existing deleterious germline mutations and influence the disease progression. This phenomenon has been so far underestimated in MPN.

In this paper I have been involved in analyzing the microarray data, as well as performed exome sequencing of the sample and subsequent validation of the identified mutations. I wrote the paper together with my supervisor, Dr. Robert Kralovics.

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LETTERS TO THE EDITOR

Rare germline variants in regions of loss of heterozygosity may influence clinical course of hematological malignancies

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Somatically acquired uniparental disomy (UPD) has been recognized as a common mechanism of mutation amplification in cancer. Somatic heterozygous mutations can become homozygous if the chromosomal arm harboring the mutation undergoes mitotic recombination and two chromosomes carrying the mutation segregate into the same daughter cell. As a result, UPD occurs, accompanied by loss of heterozygosity from the recombination breakpoint to the telomere of the affected chromosomal arm. UPD of chromosome 9p is a frequent event in myeloproliferative neoplasms and was found to be associated with the oncogenic V617F mutation of the *JAK2* gene.[1]

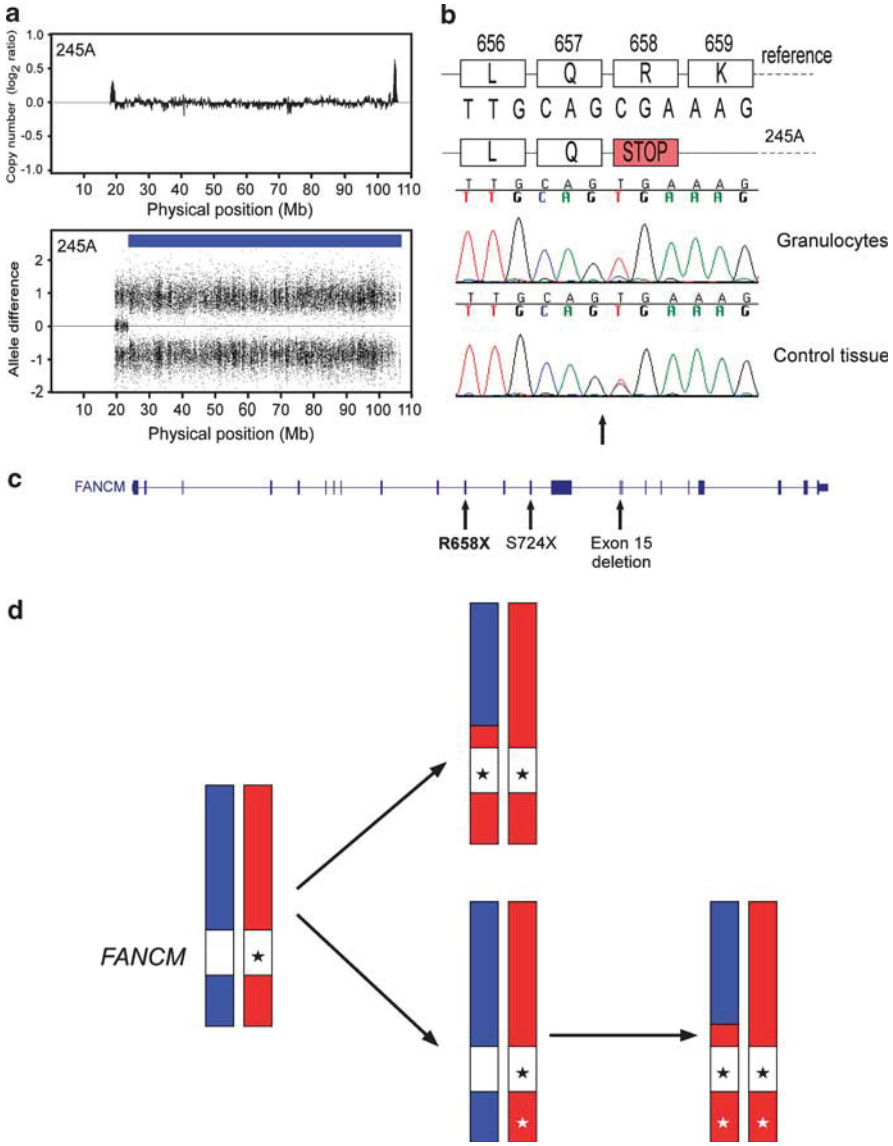

Figure 1 Homozygous nonsense *FANCM* mutation in a patient with UPD of chromosome 14q. (**a**) 14q UPD detected using Affymetrix SNP 6.0 arrays. Copy number and allelic difference data for chromosome 14 are shown. A large part of 14q shows loss of heterozygosity with no change in copy number. (**b**) R658X mutation validated by Sanger sequencing: homozygous in granulocytes (tumor tissue), heterozygous in buccal cells (control tissue). (**c**) Schematic representation of the *FANCM* gene. The mutation identified in this study is shown in bold, other two mutations described in literature are also shown.[5] (**d**) Two possible mechanisms of clonal outgrowth—either FANCM R658X is the driver or other somatic mutation that occurred on the same chromosome, drives the clonality. White star, putative somatic mutation; black star, FANCM mutation; Mb, mega base pairs.

A number of other UPDs were shown to amplify heterozygous mutations in genes such as *MPL*, *CBL* and *EZH2* in myeloid malignancies.[2–4]

We recently identified a patient with the diagnosis of polycythemia vera that carried UPD on the long arm of chromosome 14 (Figure 1a). We hypothesized that a homozygous somatic mutation might be present in the chromosomal region affected by UPD. In an attempt to identify the mutation, we employed next-generation sequencing of all the exons of the patient. Granulocyte genomic DNA was fragmented to an average of 150 bp and the synthesis of a fragment library was performed using the standard manufacturer's protocol (Illumina, San Diego, CA, USA). We enriched for exonic sequences using the SureSelect Human All Exon kit (Agilent, Santa Clara, CA, USA) and performed a 51-bp paired-end sequencing with the HiSeq2000 system (Illumina). After reference alignment of 178 million reads, variations from the genomic reference were identified and filtered based on the physical position of the 14qUPD and allelic frequency consistent with homozygosity. We further removed known single-nucleotide polymorphisms (SNPs), synonymous variants and non-coding SNPs. One of the variants validated by Sanger sequencing was a homozygous nonsense mutation in the *FANCM* gene, truncating the protein at codon 658 (R658X). We did not find R658X in any of the public databases (dbSNP, 1000 Genomes Project, OMIM, Fanconi Anemia Mutation Database). The analysis of buccal mucosa cells revealed that the patient was a germline heterozygous carrier of the *FANCM* R658X mutation (Figure 1b). Another nonsense mutation was identified in a family with Fanconi anemia of the complementation group M, where the affected pedigree members carried two mutated alleles (S724X and a 2.5 kb deletion affecting exon 15, Figure 1c).[5] As the same study also showed that the S724X nonsense mutation is a loss-of-function variant, we conclude that the more severe truncation caused by the R658X mutation is also loss-of-function. The R658X mutation is the third *FANCM* mutation reported to date.

Fanconi anemia is a heterogeneous hereditary blood disorder characterized by early onset anemia, cancer predisposition and various congenital abnormalities. The male polycythemia vera patient who carried the R658X mutation of *FANCM* was diagnosed in December 1993 at age of 44 as polycythemia vera with a hematocrit of 66.6% and serum erythropoietin levels below detection limit. The patient was positive for the JAK2-V617F mutation and required regular phlebotomies. In September 2000, the patient presented with increased leukocyte count (17.5×10^9/l). Between September and November 2003, the patient was in a plateau phase without a need of phlebotomies. In January 2004, the patient presented first time with anemia and 1% of blasts in the peripheral blood. The patient required transfusions since January 2005. We sampled the patient in September 2006 for the karyotype analysis, using the SNP 6.0 mapping arrays (Affymetrix, Santa Clara, CA, USA). The patient exhibited 9pUPD with high JAK2-V617F mutation burden and 14qUPD, but no other chromosomal aberrations. At this sampling date, the patient was anemic (hemoglobin 6.8 g/l) with 2% blasts in peripheral blood, had a leukocyte count of 25.2×10^9/l and a marked thrombocytosis (platelet count 3753×10^9/l). The blast count increased continuously (14% in June 2007), consistent with transformation to acute myeloid leukemia that was confirmed in October 2007. The patient died in April 2009.

The clinical history of the patient suggests that the patient first acquired the JAK2-V617F mutation causing the onset of the polycythemia vera phenotype. Anemia in this patient was most likely caused by a subsequent clonal selection of cells that acquired homozygosity for *FANCM* R658X due to mitotic recombination on chromosome 14q. The resulting clone homozygous for two detrimental mutations (JAK2-V617F and *FANCM* R658X) caused the transition of polycythemia to anemia, marked thrombocytosis and leukocytosis. It is possible that the leukemic transformation in this patient was a consequence of the homozygosity to the *FANCM* mutation, as Fanconi anemia patients often develop acute leukemia. At this point, we do not know if homozygosity for R658X was driving the clonal selection. It is possible that a somatic mutation on 14q was the driver and the *FANCM* R658X mutation co-acquired homozygosity with the yet unknown driver mutation (Figure 1d).

In a recently published high-resolution SNP microarray study, we detected 147 UPD events affecting 12 different chromosomal arms in 321 chronic phase myeloproliferative neoplasms patients (9pUPD was present in about 37% of myeloproliferative neoplasms cases).[6] All these UPD events could switch rare deleterious germline variants to homozygosity and influence the clinical phenotype of patients. We performed exome sequencing in 11 patients with 9pUPD. In average, we detect 636 non-synonymous variants per patient that are not in public databases (dbSNP, 1000 Genomes Project). The 9pUPD regions of these 11 cases contained between 0–7 non-synonymous variants per

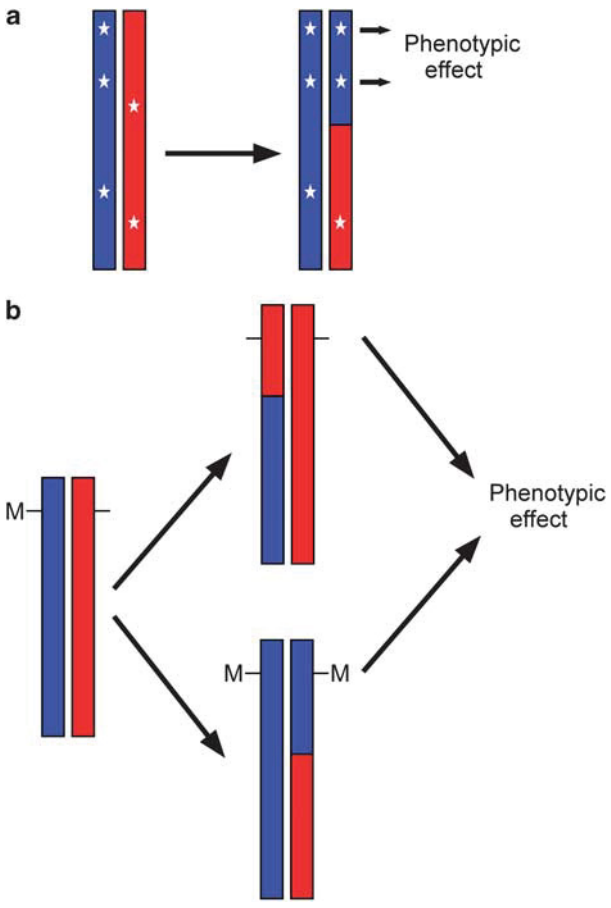

Figure 2 Possible mechanisms of interaction between somatic and germline genetic factors. (**a**) Alleles underlying germline recessive traits are made homozygous by somatic UPD influencing the clinical phenotype. (**b**) Imprinted or monoallelically expressed genes are affected by somatic UPD. As a result, increased or reduced gene expression may occur. White stars, mutations; M, DNA methylation of an imprinted gene.

patient (excluding JAK2-V617F). At this point, it is challenging to predict the influence of these non-synonymous variants on the clinical phenotype of each individual patient. However, using the PolyPhen-2 algorithm (http://genetics.bwh.harvard.edu/pph2), one third of these non-synonymous variants are predicted to damage the protein function (data not shown). Therefore, any myeloproliferative neoplasms patient with loss of heterozygosity has the potential to embark on a unique clinical course driven by the distinct set of rare variants each patient carries within the loss of heterozygosity region.

In general, somatically acquired UPDs can influence phenotypic effect of germline variants and epigenetic marks (Figure 2). UPDs can result in homozygosity of rare deleterious germline mutations (Figure 2a) that have no phenotypic effect when heterozygous. In addition, UPDs may either increase or reduce the expression of imprinted genes located on the affected chromosomal arm (Figure 2b). Although the prevailing opinion in cancer genetics is that somatic mutations are contributing to disease initiation and progression, our study demonstrates that a unique interplay of germline variants with somatic aberrations may influence disease pathogenesis in each individual case.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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MicroRNA signatures characterize multiple myeloma patients

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MicroRNAs (miRNAs) are a class of small non-coding single-stranded RNAs of ~22 nucleotides in length that regulate protein levels by binding to either partially or complete complementary sites in messenger RNAs (mRNAs), leading to translational repression or transcript degradation, respectively. MiRNAs have a role in critical biological processes including cellular growth and differentiation. Recent studies showed that miRNAs have an important role in the pathogenesis of multiple myeloma (MM) and that miRNA signatures are associated with different cytogenetic subtypes. Unsupervised analyses of miRNA expression in MM identified unique clusters, which were not associated with chromosomal abnormalities, while supervised analysis showed a specific miRNA expression pattern for MM subgroups.[1–3]

We have investigated the expression level of 365 miRNAs in sorted bone marrow (BM) plasma cells of 45 newly diagnosed MM patients enrolled in the HOVON-65/GMMG-HD4 trial (Supplementary Table S1) and 4 healthy individuals (Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/, accession number GSE16558).[1] We addressed the question whether MM patients could be classified based on miRNA expression patterns. MiRNA expression data were linked to gene expression data to gain more insight into candidate miRNA target genes. Finally, a possible association of miRNA expression with response to therapy, that is, complete response (CR), nearly CR (nCR), progression-free survival (PFS) and overall survival (OS), was evaluated.

Unsupervised hierarchical cluster analysis based on miRNA expression showed that normal BM samples clustered distinctly from MM samples (Figure 1). A total of 44 MM samples were grouped in four distinct clusters and one MM sample was not included in any MM cluster. The robustness indices per cluster are shown in Supplementary Table S2. To determine the miRNA expression signatures of the four MM and one normal BM cluster, each cluster was compared with the remaining clusters using the Mann–Whitney *U*-test with a false discovery rate (FDR) <0.05 (Partek Genomics Suite 6.4 software). The top 10 significant distinctive miRNAs for the four MM clusters and normal BM cluster are shown in Tables 1 and 2, respectively. The complete list of differentially expressed miRNAs (FDR <0.05) characterizing the four MM clusters and normal BM cluster is shown in Supplementary Tables S3 and S4, respectively. MM cluster 1 ($n=8$) was characterized by upregulation of 17 miRNAs, including *miRNA-130a*, *-424* and *-622*; MM cluster 2 ($n=9$) by upregulation of *miRNA-576* and *-106b*; MM cluster 3 ($n=4$) by upregulation of 23 miRNAs, including *miRNA-372* and *-200a*, and downregulation of *miRNA-146b*; and MM cluster 4 ($n=23$) was characterized by downregulation of 60 miRNAs. An interesting observation is the

4. DISCUSSION

The work performed in the context of my thesis resulted in several outcomes important for both MPN research field and human genetics in general.

SEGEX. We developed an algorithm for familial linkage analysis which can be useful to study any inherited disorder. There is no need to specify the mode of inheritance and penetrance in SEGEX analysis. This is an advantage for many complex diseases, for which the exact level of penetrance is not known. Although from the first sight it seems that SEGEX will call too many shared regions, in practice with the use of large number of SNPs the opposite is true. With the SNP density currently offered by the SNP arrays most of the genome is excluded and only the regions which are truly shared between affected members, i.e. which are identical by descent (IBD), remain, which is the ultimate goal of all allele-sharing analysis methods.

The three families used for validation of the method showed that the method can efficiently identify the shared genomic regions which contain causative mutations. In two of them, the one with Parkinson's disease and the first one with hereditary thrombocytosis, only a single region was identified with linkage to the disease and the causative mutation was located in that region. The analysis of the third family, with hereditary thrombocytosis, detected three regions and one of them contained the mutation causing the disease. Overall, the results from the study of these three families prove that the method correctly finds the regions in linkage. In summary, described method is simple and efficient for non-parametric linkage analysis of hereditary disorders. Similar methods could be developed for parametric linkage analysis, which will also use genotype information from unaffected members of the family. However, there are factors such as low penetrance and late age of onset, as well as the effect of environment, which are quite frequent for complex diseases that make the use of parametric tests non-practical, if not impossible at all.

We observed strong and inverse correlation between the meiotic count, i.e. number of meioses separating all the available affected members of the family and the total size of linkage regions detected. With sufficiently large family, namely with 10 or more meiotic count, it is highly likely to find only a single region in linkage and with currently used DNA sequencing technologies it is feasible to sequence a region of 10-20 Mb to identify the causative mutation.

Parametric and non-parametric linkage tests still remain cornerstones of familial linkage analysis. At the same time, technological advances in genetics and availability of new methods of data generation provide basis for the development of conceptually new methods of analysis. The high density of SNP markers and accuracy of genotyping are two key factors ensuring the efficiency of this method. Affymetrix Genome-wide Human SNP 6.0 arrays seem to be suited for this method quite well, having rather high marker density and low false genotype call rate. We have tried to utilize this method for other types of microarrays, however, for some the marker density was low (Affymetrix 10K, 50K); for others, although density was enough, the genotyping accuracy was not high enough (Affymetrix 250K), which resulted in lower accuracy in calling shared regions.

Current trends in analysis of familial diseases are going towards even more simplification, there are already a few publications where whole genome sequencing of the affected family members has been performed and causative mutations have been identified (Zimprich et al, 2011). Though we think that familial linkage analysis is still necessary in order to reduce enormous complexity of the whole genome sequencing data.

RBBP6. Another outcome of my thesis is the discovery of germline mutations predisposing to MPN. We have identified germline *RBBP6* mutations in about 5% of familial MPN cases and in about 0.6% of sporadic MPN cases where family history is unknown. The low penetrance present in MPN pedigrees suggests that the disease is triggered by some stochastic factors. As the phenotype in familial MPN is driven by somatic mutations, the acquisition of somatic mutations may be the factor that influences penetrance. It is likely that mutant RBBP6 causes an elevation in somatic mutagenesis rates through inhibition of p53 function. The p53 pathway has recently been shown by our group and others to play a crucial role in the MPN pathogenesis (Harutyunyan et al, 2011b; Nakatake et al, 2012). Since the hematopoietic tissues exhibit high mitotic activity, hematopoietic cells may be the most affected by elevated mutagenesis rates. In this case studying somatic and germline genetics of MPN helped us to pinpoint the key role of p53 pathway in MPN. As *RBBP6* is widely expressed, the question as to how *RBBP6* mutations cause predominantly MPN phenotype remains elusive. There are examples of germline mutations in cancer associated genes causing a specific familial phenotype, e.g. retinoblastoma (*RB1*) (Lee et al, 1987), neurofibromatosis (*NF1*) (Wallace et al, 1990), melanoma

(*CDKN2A*) (Hussussian et al, 1994) and others. Similarly, germline *RBBP6* mutations may predispose toward myeloproliferative phenotypes. Our results show that apart from the weak MPN susceptibility exerted by a common *JAK2* haplotype (Jones et al, 2009; Kilpivaara et al, 2009; Olcaydu et al, 2009a), other germline factors with higher penetrance confer predisposition to MPN. The *RBBP6* mutation frequency of 5% in MPN pedigrees underlines the genetic heterogeneity of susceptibilities to myeloproliferative phenotypes.

***JAK2* 'GGCC' haplotype.** Besides familial strong predisposition, there are also common alleles in population that confer weaker susceptibility to MPN. In parallel with two other groups we have found the strongest population-wide MPN predisposition, the haplotype around *JAK2* gene (Olcaydu et al, 2009a). This risk allele frequency is about 30% in the European populations, while the odds ratio (OR) for MPN is about 2.5. The very high OR implies that it is unlikely that any other similar common predisposition for MPN will be found. Importantly, there is the strong evidence that 'GGCC' haplotype predisposes only to JAK2-positive MPN, despite some reports claiming *JAK2* mutation-independent MPN predisposition (Pardanani et al, 2010b; Tefferi et al, 2010b). This in turn means that there might be other risk loci for JAK2-negative MPN (although most likely weaker).

We also examined the role of *JAK2* haplotype in familial MPN cases since it was hypothesized that the haplotype might explain familial clustering in MPN. As a result of our study, we excluded such a possibility, showing that there is no strong enrichment of the risk haplotype in familial MPN compared to sporadic MPN (Olcaydu et al, 2011). On the other hand, we have demonstrated that the 'GGCC' haplotype still confers the same risk of developing JAK2-positive MPN in familial cases as it does in sporadic cases (Olcaydu et al, 2011). This goes in line with the hypothesis that the germline variants predispose to acquisition of various somatic mutations which then drive the disease.

Rare germline alleles and LOH. The influence of germline factors on MPN pathogenesis is even more complex. We have described another mechanism through which germline variants can exert their effect (Harutyunyan et al, 2011a). There are a number of recessive deleterious germline variants in each individual. When these variants accidentally become homozygous or hemizygous as a result of a somatic LOH event spanning that chromosomal region, they might start to influence the disease phenotype. It will depend on tissue and cell-type gene expression and

environment whether the variant will influence disease pathogenesis, but the example of *FANCM* and 14q UPD that we have described shows that such interaction of somatic and germline genetics can be very relevant in cancers (Harutyunyan et al, 2011a).

4.1 Recent findings in MPN germline genetics

Currently there is a lot of research activity in MPN germline genetics field. Since the discovery of *JAK2* haplotype conferring MPN susceptibility there have been numerous reports assessing the role of *JAK2* haplotype in different contexts (Jones et al, 2010; Olcaydu et al, 2009b; Pardanani et al, 2010b; Tefferi et al, 2010b), as well as trying to identify new predisposition factors.

Most of the germline SNPs recently described have not been studied in large number of patients and are often controversial. Possibly the most robust association found is for a coding SNP (K751Q) in *ERCC2* (or *XPD*) gene associated with leukemic transformation in MPN (Hernandez-Boluda et al, 2012). The association was shown for PV and ET patients (5-fold increased risk for leukemic transformation) while examining the polymorphisms in several DNA repair genes. *ERCC2* is involved in nucleotide excision repair pathway and the mutation likely impairs DNA repair capabilities in those patients thus predisposing to acquire more mutations and transform to sAML. Another study examined the same polymorphism in PMF and came to the conclusion that this SNP does not play a role in leukemic transformation of PMF patients (Poletto et al, 2013). The authors suggested that there are different mechanisms involved in leukemic transformation in PMF compared to PV and ET. However, when we look at the actual data, even in PMF samples there is a trend for SNP association with leukemic transformation (2-fold difference) and p value is just above the threshold of 0.05 (Poletto et al, 2013). So it is more plausible that *XPD* polymorphism confers risk to develop sAML in all three MPN subtypes and the reason the p value in their study was not significant might be just due to small sample size. The identification of a polymorphism influencing the disease progression in MPN can have significant implications in patient management and the choice of therapy. This fact emphasizes the need to keep germline genetics in focus for MPN.

Several other associations with MPN have been reported although all of those are disputed and should not be taken for granted. Following up on the report of

CCDC26 polymorphisms being associated with *IDH1* and *IDH2* mutations in gliomas (Jenkins et al, 2011), a research group tried to replicate those findings in MPN (Lasho et al, 2012). They found the inverse association of the described SNP with acquisition of *IDH1/2* mutations. However, due to small sample size and inverse association these findings are likely not to be confirmed in other cohorts. Indeed, there have been no follow ups on this matter.

Another group reported on association of a polymorphism in glucocorticoid receptor gene (*GR* or *NR3C1*) with MPN (Varricchio et al, 2011b). The authors have followed up and checked for association in Diamond-Blackfan anemia (Varricchio et al, 2011a), and different aspects of MPN pathogenesis (Poletto et al, 2012). However, all the reports on this polymorphisms come from the same group; no confirmatory studies have been done by others. Additionally, when looking on the actual study design, the case and control populations are not properly matched, mainly with a different ethnic background. And this in case when even the authors are mentioning in the text that there is huge inter-population variability for this SNP. Unless these findings can be independently confirmed by other groups in larger number of patients, they cannot be truly implicated in MPN pathogenesis.

And to mention some negative results of association studies. There was a report showing no association of *MDM2* polymorphism with MPN (Rumi et al, 2012a). Also no association for MPN was found with *TP53* serine 72 polymorphism (Raza et al, 2012).

The familial MPN research has had fewer successes recently. Perhaps due to extensive heterogeneity researchers are consistently failing to identify or confirm mutations in familial cases of MPN. Other than *RBBP6* reported in this thesis, no other genes have been implicated in familial MPN.

There are more and more results being available on germline MPN genetics. As it becomes apparent, inherited polymorphisms not only simply confer risk to develop the disease, but they also influence various aspects of the pathogenesis. With the elucidation of the role of germline genetics, a more complete picture of MPN, as a complex interaction of germline and somatic factors, should emerge.

4.2 Genes mutated somatically and germline

An interesting aspect of MPN genetics is the overlap between the somatic and germline genetics. This relates to the mutations that can occur in the same gene both somatically and germline. This is a well-known phenomenon extensively studied in cancers and in many cases the impact of mutations in the same gene is not exactly identical when it occurs somatically or is inherited germline (Hussussian et al, 1994; Lee et al, 1987). The reasons for this can be both the length of exposure to the mutation (from zygote in germline compared to much shorter time for somatic mutations) and the context (all cells have the mutation in case of germline, so they have no selective advantage, compared to “uniqueness” of the cell carrying the somatic mutation).

Regarding the same phenomenon in MPN, the most MPN-specific mutations are those in *JAK2* and *MPL*. These genes can also be mutated germline and lead to the development of hereditary thrombocytosis, an MPN-like disorder (Ding et al, 2004; Mead et al, 2012). A more complex but similar example are the mutations in *CSF3R*. Inherited loss-of-function *CSF3R* mutations cause neutropenia while somatic gain-of-function *CSF3R* mutations are commonly found in chronic neutrophilic leukemia (Beekman et al, 2013; Maxson et al, 2013). There are other examples in myeloid malignancies (including MPN) of genes that can mutate both somatically and germline, such as *CBL* and *RUNX1*. The first cause developmental symptoms and JMML (Niemeyer et al, 2010), while the second cause thrombocytopenia with susceptibility to develop AML (Song et al, 1999). Interestingly, in the latter cases the patients do acquire additional somatic mutations and follow the classical multi-step cancer evolution. All these examples demonstrate that many of the MPN-related genes are involved in both germline and somatic context. That in turn suggests that finding a gene somatically mutated in MPN can imply its possible role also as a hereditary factor and vice versa.

4.3 Phenotype-specific germline mutations

JAK2-V617F mutations are found in all three subtypes of MPN, however, it is not well understood how can the same mutation cause different phenotypes. Several explanations have been suggested, such as the influence of other somatic mutations

or germline factors. So far no consistent somatic mutational pattern has been observed to explain the phenotypic diversity. The low number of discovered germline factors does not allow assessing their effect on disease phenotype. Another possibility that has been brought up is the dosage of JAK2-V617F mutation. It has been noted in numerous studies that 9p UPD following JAK2-V617F mutation is rather frequent in PV and to a lesser extent in PMF, but is almost absent in ET (Klampfl et al, 2011; Stegelmann et al, 2010). Additional evidence comes from JAK2-V617F transgenic mouse models. The mice with higher dosage of V617F develop PV-like disease, while those with lower copies of JAK2-V617F display ET-like phenotype (Tiedt et al, 2008; Wernig et al, 2006; Zaleskas et al, 2006). Overall these facts may suggest that the higher the *JAK2* mutational burden, the higher the likelihood to develop PV and the lower the chance of ET. It is also suggested that JAK2-V617F mutation is embryonically lethal and thus cannot be inherited (Marty et al, 2010). On the contrary, other *JAK2* mutations (V617I, R564Q, H608N) were found to be germline (Etheridge et al, 2011; Mead et al, 2012; Rumi et al, 2012b), suggesting that these are milder mutations than V617F. And accordingly, germline *JAK2* mutations cause the mild phenotype of hereditary thrombocytosis. Recently it has been shown that in there are differences in JAK-STAT pathway activation between JAK2-positive ET and PV: STAT1 phosphorylation is higher in ET, while STAT5 phosphorylation is higher in PV (Chen et al, 2010). In conclusion, *JAK2* mutation type, mutation dosage and possibly other germline and somatic factors contribute to the phenotypic determination of MPN.

Unlike the significant phenotypic diversity of MPN in the presence of similar mutational profile, the phenotypic features of hereditary diseases with *JAK2* and *MPL* mutations are rather restricted. In case of germline *JAK2* mutations, it is hereditary thrombocytosis, similar to *MPL* mutations, although in the latter case the onset is in childhood. This extreme specificity of the clinical features of hereditary MPN-like diseases is partly due to the fact that the disease is driven by the germline mutation and not by additional somatic mutations. But similar thing might exist for true MPN cases, since it was shown in other cancers that transcriptional profile correlates with the predisposing germline mutation (Comino-Mendez et al, 2011). Therefore in order to facilitate the search for familial MPN mutations, the families should be grouped according to their clinical and molecular parameters which in the end may result in separation of specific familial MPN subtypes.

4.4 Concluding remarks and future directions

The field of MPN germline genetics is advancing fast. In the last few years a number of new findings are starting to uncover the hereditary factors involved in MPN pathogenesis. This has been undoubtedly assisted by the remarkable technological advances in the molecular genetics field, such as microarray genotyping and second generation sequencing. Currently there have been a number of germline genetic factors identified, ranging from some with rather high frequency in the population but lower penetrance and on the other end of the spectrum very rare but highly penetrant mutations clustered in families (**Figure 21**).

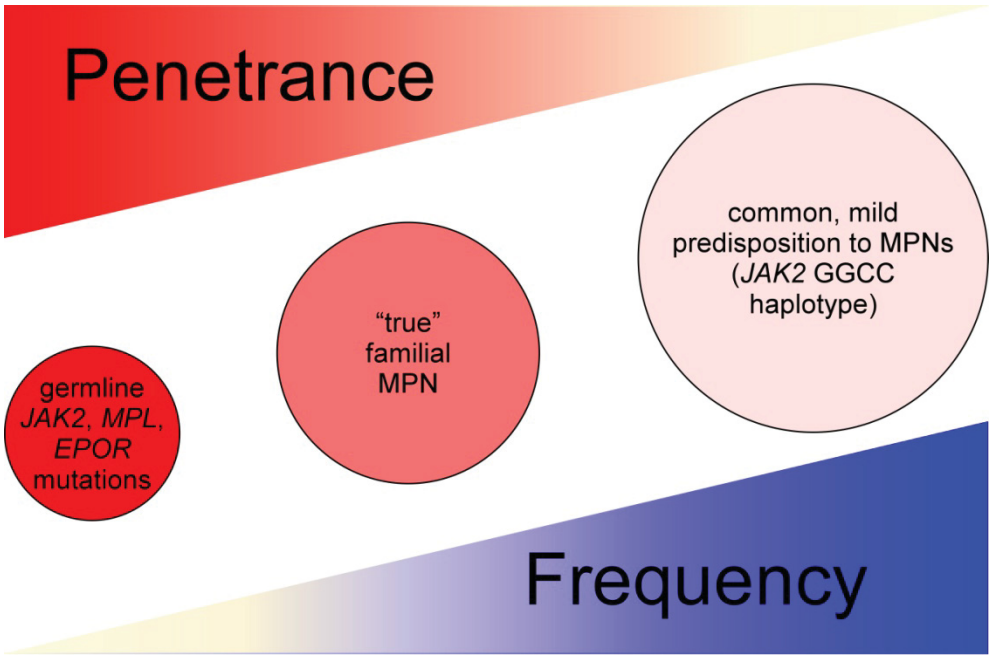

Figure 21. Germline predisposition to MPN and MPN-like diseases. The shades of the red indicate the level of penetrance (darker corresponds to higher), while the size of circles displays the frequency of the variant. (*Figure reprinted from Hematology/Oncology Clinics of North America 26, Harutyunyan A., Kralovics R. Role of Germline Genetic Factors in MPN Pathogenesis. 1037-1051. Copyright 2012, with permission from Elsevier*)

All of these findings combined with the new discoveries in somatic genetics of MPN allow us to envisage the pathogenesis of MPN as a multidimensional interaction of somatic and germline mutations and other factors. This overview of MPN pathogenesis is not yet complete and new findings will more precisely define each step of pathogenesis and allow us to obtain a more differentiated categorization of

MPN subtypes based on molecular markers. As a consequence, a proportion of patients will be reassigned to MPN-like diseases category as it happened for patients diagnosed with ET and carrying germline *JAK2* mutations. The current view of MPN pathogenesis is represented on **Figure 22**.

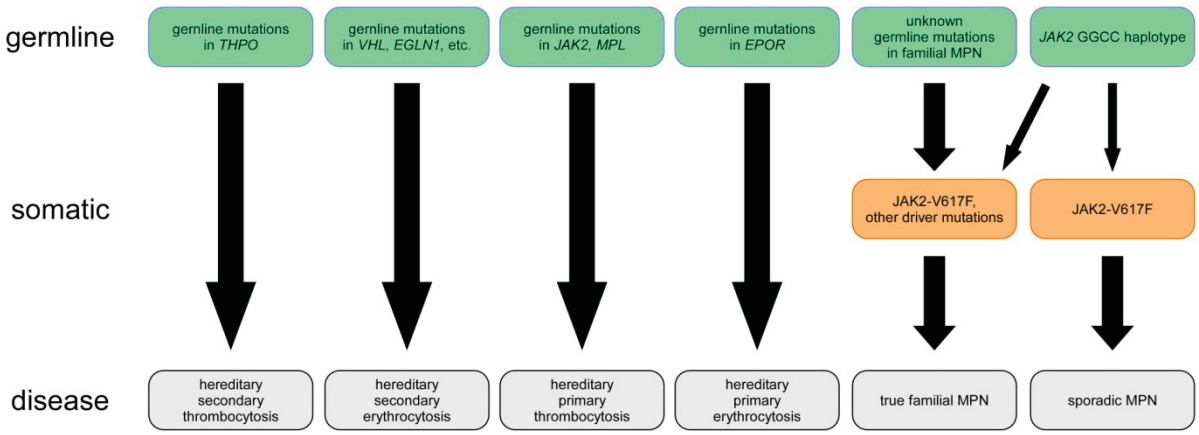

Figure 22. Current understanding of the pathogenesis of MPN and MPN-like disorders. (*Figure reprinted from Hematology/Oncology Clinics of North America 26, Harutyunyan A., Kralovics R. Role of Germline Genetic Factors in MPN Pathogenesis. 1037-1051. Copyright 2012, with permission from Elsevier*)

So far the main focus of MPN genetics research has been on protein coding regions of genes, but this is going to change soon. With the more widespread use of second generation sequencing not only for genome sequencing but also for other purposes such as bisulphite sequencing, ChIP-seq and RNA-seq, the role of non-coding variation and epigenetic modifications in MPN pathogenesis will start to shape.

5. MATERIALS AND METHODS

5.1 Materials

Patient samples. Peripheral blood samples were collected from familial and sporadic MPN patients after written informed consent at the Medical University of Vienna, Austria, the University of Pavia, Italy, and Royal Perth Hospital, Perth, Australia, according to the regulations of the local ethics committees. Healthy control samples were obtained from Italy (n=203) and Central Europe (n=520). Genomic DNA was isolated from granulocyte and T-cell cell fractions of these blood samples according to standard procedures.

Affected members from three families have been chosen for validating the SEGEX algorithm. All three families had been previously studied by parametric linkage mapping methods and the causative mutations have been identified. We included one pedigree (6 subjects) with Parkinson's disease with a mutation in *LRRK2* gene (Zimprich et al, 2004), and two pedigrees (a total of 18 individuals) with hereditary thrombocythemia with a mutation in *THPO* gene (Liu et al, 2008; Wiestner et al, 1998). Peripheral blood samples were collected after written informed consent of the studied subjects. The study protocols were approved by the local ethics boards.

5.2 Methods

Microarray genotyping. DNA samples were processed and hybridized to Genome-Wide Human SNP 6.0 arrays (Affymetrix) according to the protocol supplied by the manufacturer. The raw data was analyzed by Genotyping Console version 3.0.2 software (Affymetrix). The samples were assessed for chromosomal aberrations (deletions, gains and acquired uniparental disomies) as implemented in the Genotyping Console software. Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix) allows genotyping of 909,622 SNPs. The genotype data with positional information has been exported in AA/AB/BB format as a tab-delimited text file for further analysis.

Exome sequencing. An equimolar pool of DNA samples from three affected

family members was prepared, processed and enriched for exons using Agilent SureSelect Human All Exon Kit (Agilent) according to the protocol supplied by the manufacturer. DNA libraries were sequenced using 51-bp paired end sequencing on an Illumina HiSeq 2000 system. Image analysis and base calling was performed using Real Time Analysis 1.12 software (Illumina). Resulting BCL files were converted to FASTQ format with the CASAVA 1.7 software (Illumina). Alignment of the reads and subsequent SNV and DIV calling were performed using CLC Genomic Workbench 4 software (CLC bio). For alignments, a maximum of 2 mismatches or 4 bp insertions or deletions were allowed. Variants were called that had coverage of at least 10 and frequency of 25%. The SNV and DIV lists were exported as text files, converted to MAQ and GATK bed formats respectively and uploaded to SeattleSeq SNP Annotation Server web tool (http://gvs.gs.washington.edu/SeattleSeqAnnotation/). The annotated SNVs and DIVs were filtered by excluding variants which were in dbSNP129 or 1000 Genomes Project database, for non-coding and synonymous variants. Finally, focus was on the variants in the genes which were in the shared genomic regions identified by SEGEX linkage analysis (**Table 2**).

Sanger sequencing. All primer sequences and PCR conditions used for the study described in section 3.2 are provided in the **Tables 6 and 7**. Exon sequencing of *RBBP6* and validation of mutations from exome sequencing data was performed using the BigDye Terminator v3.1 Cycle Sequencing kit and the 3130xl Genetic Analyzer (Applied Biosystems). Sequence analysis was done using the Sequencher Software 4.9 (Gene Codes).

Allele specific PCR. *JAK2* mutational status was determined using allele specific PCR for the JAK2-V617F mutation as described elsewhere (Kralovics et al, 2006). All the primer sequences and PCR conditions for AS-PCRs of detected mutations are listed in **Table 8**. Wild type and mutant alleles were amplified with allele-specific dye-labeled primers of different length and a common reverse primer. PCR product length differences were detected on the 3130xl Genetic Analyzer (Applied Biosystems). The data were analyzed using the Gene Mapper 4.0 software (Applied Biosystems).

Table 6. Primers and PCR conditions for sequencing.

gene	exon	Forward primer (5' -> 3')	Reverse primer (5' -> 3')	PCR conditions
RBBP6	1	GGTCCTTCGGTGTCTTTGAG	AGAGACATAATGGCGGTTAGC	
	2	GACAATATCAGTAATCTAGTTGCAAGG	CGAACAAAGAAACCTACTATCCTC	
	3	TCAAAGGTGGTAAAGATTAATCAGAG	AAGTGTGCAAGTGATGCCAC	
	4	TCTAGAGAAGAATGAAGTAGGCTGAG	TTGCAAAATATGTCCTCCCC	
	5-6	GATACTGCTCTTTACAGGATGAGTG	GCCATTGTCTACAGAAGGCAG	
	7	GTTCTGGCACTTGGCTGAG	AGCAGACTGCAGCCTCACAG	
	8	ACTAATTTGGCATTGCGCTG	AAACAGACTTCTCACTACTTAATGCTC	
	9	AACTGAACAAATGTGGACCATC	TTTACAGCCTACAGATACCAAGC	95°C-5 min.
	10	TCTCCTGATGCTTTAATTGCC	TCATCCCACATTACTGAGCAC	*10x touch down*
	11-12	TGCAGGTGATGGGTAAACAG	CCTGGACGAGCAGTATTTATTC	*(-1°C/cycle):*
	13	GGTGAGTAAGATCACTTTGGTTTAG	TGCCTAGTTTGGACCTTCC	94°C-30 sec.
	14	CGAAACAAATTTAAGAACTGGGG	GCACAATACAAACAAATGACCC	67°C-57°C-30 sec.
	15	TTGAATAGTTGGTTCTATTCCACTG	TGGCTTTGGTTTAAATTTTAGGTG	72°C-30 sec.
	16	CAAGCAACTTCTCAGGGATTC	TCTTCACTTCTATACACCTAGACCG	*26x:*
	17_1	TGAAACCGTGAGGAAAGAATG	TCTCTCCCATTCTCGGTAGC	94°C-30 sec.
	17_2	CTCAAGCGTTTAGGGGACAG	CACTTTCCTCCCCTTTTCTTC	57°C-30 sec.
	17_3	TCGAAACATAGGTAGCAACTATCC	TTTAATTGGAGGTTCAGATCGAG	72°C-30 sec.
	17_4	AAACCAAAAGCAAAGGGTG	ATTTTGCGCTTTGGTGATG	72°C-10 min.
	17_5	AAAGGCCAAGAAGCCTAATG	CCTACTTCCCTAGATCAGCATTC	
	18_1	TTGTGTGTCTTCCTTAGGGC	TCTCTGGCTGAGTGCTGTTC	
	18_2	AATTCACCAAGGACGTGAGC	TTTGTCTCTTCATTTGGCCG	
	18_3	CCTTCTCGGAATAAAGATTCTGC	TATGCCCACCTGGACAACTG	
	18_4	TGATGAAGCTGCTTTTGAACC	AAGTGCAGCACAGGGTCTTC	
JAK2	12	CAAAGTTCAATGAGTTGACCCC	TGCTAACATCTAACACAAGGTTGG	
	14	GCTGAAAGTAGGAGAAAGTGCATC	CTGACACCTAGCTGTGATCCTG	
MPL	10	AGAGTAGGGGCTGGCTGG	AGGTGACGTGCAGGAAGTG	

Abbreviations: min., minute; sec., second.

Table 7. Primers and PCR conditions for validation of mutations by Sanger sequencing.

Gene	Mutation	Forward primer (5' -> 3')	Reverse primer (5' -> 3')	PCR conditions
ABCE1	P243A	CCTGGTGTGTAATCTTGCTTTCT	GGTGGCAGCTATAAACAACCA	
VEGFC	M418del	TTTGTTAGCATGGACCCACA	TGATGAATCCATTGCCTTGA	
FRG1	E55G	GCAAGATTTAATCGAGACAAAGTG	CAATGGTTCCATTCCTTTGG	
PARP4	I1039T	GTGGTGGTGTACGCCTGTAG	AAATCTGCTGGTGAGAAGGG	
CCNA1	V252GfsX29	TTTCCAACCTTTGCTTGTGA	AGCTGCAGTTTCCCTCTCAG	95°C-5 min.
AHNAK2	Q265X	GCTTGTAGGGGACACGTCAT	CCACGAAAACTCTGGAAGGA	*10x touch down*
NDE1	R234H	TTATAAACATGAGCCACTGCG	CGTAGCTGCAGAGTGGCAG	*(-1°C/cycle):*
RBBP6	R1569H	CGAAAAGACTCTCCTTCTCGG	TGGTTCAAAAGCAGCTTCATC	94°C-30 sec.
ARMC5	P507L	TTGGCTCTGGGTTCAGTCTC	ATAGGTCAGCAGGCCGTACA	67°C-57°C-30 sec.
CACNG4	S184A	CATCAGAGAGGGGAGTGTCC	CCCTGTGATCTTCAGGCCC	72°C-30 sec.
C20orf3	D395N	GGTGAACTTTCCCTGCACTG	ATGTGGACACTTGAACCACG	*26x:*
TP53TG5	N97del	ACCTGGGACTTCCACTCCTT	TGCTCATCACACCTCCTCAC	94°C-30 sec.
C20orf123	T163N	AAACAGCCCTGTGACCACTC	CCTCCTGGTCTTCCTGAGC	57°C-30 sec.
FAM65C	D318G	GAGTTCAAGGATGCAGTGAGC	CTCAAACGGAAGCTGAGCC	72°C-30 sec.
ARSD	X383Q	CAAAATCAACCTTCTACACAGCC	TGAACTCCTGGTCTCAAGCC	72°C-10 min.
ZXDB	G130D	AGGACCGATCAACCTAGCG	CGTTCTCAAAGCGCAACAG	
AR	Y483X	CTCTTCACAGCCGAAGAAGG	GTAGCTATCCATCCAGGGGC	
MSL3L1	A174S	CCCTCCTGGAGTAGAGAGAGC	AACACAAAGTACACGCTGGC	

Abbreviations: min., minute; sec., second; fs, frameshift; del, deletion.

Table 8. Primers and PCR conditions for mutation screening by AS-PCR.

Gene	Mutation	Forward primer (5' -> 3')	Reverse primer (5' -> 3')	PCR conditions	
RBBP6	R1569H	A:**6-FAM**-AAAATAAAAATCCTTGTAAGGATCA G:**6-FAM**-ATAAAAATCCTTGTAAGGATCG	ACAGTACTCTTGTCAATTTGCC	55.5°C	
RBBP6	E1654G	CCACCAGAGACACAGGTTGA	A:**6-FAM**-AATGTTTCCTGAAGATTCCTCTT G:**6-FAM**-TTTAATGTTTCCTGAAGATTCCTCTC	62.2°C	
RBBP6	R1451T	AGGGAAAACCAAAGATCGAGA	G:**6-FAM**-GTGGAATCATGTTTATCTGACGTTC C:**6-FAM**-TTGTGGAATCATGTTTATCTGACGTTG	65.0°C	95°C – 5 min.
RBBP6	S1444F	C:**6-FAM**-ATGAACAAGGAAATTTTAAAAGTCTGTC T:**6-FAM**-AATATGAACAAGGAAATTTTAAAAGTCTGTT	TGCAGAATCTTTATTCCGAGAAG	61.7°C	*32-34x:* 94°C-30sec.
RBBP6	I1661V	A:**HEX**-AGAAGAGGAATCTTCAGGAAACA G:**HEX**-TTTAGAAGAGGAATCTTCAGGAAACG	CTGAGCTGGCACTGCTACTG	62.6°C	anneal-30sec 72°C-30sec
RBBP6	A1673V	C:**HEX**-CCTGAAAGATAAAATAGTGGAGAAAGC T:**HEX**-TTTCCTGAAAGATAAAATAGTGGAGAAAGT	CTGAGCTGGCACTGCTACTG	60.0°C	72°C-15 min.
C20orf3	D395N	CCACCACCTCTCAGGGACTA	C:**6-FAM**-GCGAGGTGCACGAACACG T:**6-FAM**-AAAGCGAGGTGCACGAACACA	68.8°C	
ARMC5	P507L	C:**6-FAM**-ACGCACCCAACGCACTCC T:**6-FAM**-AAAACGCACCCAACGCACTCT	ATAGGTCAGCAGGCCGTACA	65.3°C	

Abbreviations: min., minute; sec., second; 6-FAM, carboxyfluorescein; HEX, hexachloro-fluorescein.

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7. ABBREVIATIONS

6-FAM	carboxyfluorescein
AAS	absence of allele-sharing
ABL1	c-abl oncogene 1 (gene)
aCML	atypical chronic myeloid leukemia
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
ARMC5	armadillo repeat containing 5 (gene)
ASXL1	additional sex combs like 1 (gene)
Balb/c	mouse strain
BCR	breakpoint cluster region (gene)
BPGM	2,3-bisphosphoglycerate mutase (gene)
BR	binding region
C20orf3	chromosome 20 open reading frame 3 (gene)
C57Bl/6	mouse strain
CBL	Casitas B-lineage lymphoma proto-oncogene (gene)
CCDC26	coiled-coil domain containing 26 (gene)
CDKN2A	cyclin-dependent kinase inhibitor 2A (gene)
cDNA	complementary DNA
CDR	commonly deleted region
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha (gene)
CGH	comparative genomic hybridization
chr	chromosome
ChIP-seq	chromatin immunoprecipitation with sequencing
CML	chronic myeloid leukemia
CMML	chronic myelomonocytic leukemia
CSF3R	colony stimulating factor 3 receptor (granulocyte) (gene)
CUX1	cut-like homeobox 1 (gene)
del	deletion
DIV	deletion insertion variant
DNA	deoxyribonucleic acid
DNMT3A	DNA cytosine methytransferase 3 alpha (gene)
DWNN	domain with no name

EGLN1	egl nine homolog 1 (C. elegans) (gene)
EPAS1	endothelial PAS domain protein 1 (gene)
EPOR	erythropoietin receptor
ERCC2	excision repair cross-complementing rodent repair deficiency, complementation group 2 (gene)
ET	essential thrombocythemia
EZH2	enhancer of zeste homolog 2 (Drosophila) (gene)
FANCM	Fanconi anemia complementation group M (gene)
FGFR1	fibroblast growth factor receptor 1 (gene)
FLT3	fms-related tyrosine kinase 3 (gene)
fs	frameshift
GATA2	GATA binding protein 2 (gene)
GR	glucocorticoid receptor (gene)
HBB	hemoglobin, beta (gene)
Hg1	human genome assembly 18
HEX	hexachloro-fluorescein
IDH1	isocitrate dehydrogenase 1 (NADP+), soluble (gene)
IDH2	isocitrate dehydrogenase 2 (NADP+), mitochondrial (gene)
IKZF1	IKAROS family zinc finger 1 (gene)
JAK2	Janus kinase 2 (gene)
JMML	juvenile myelomonocytic leukemia
L3MBTL1	l(3)mbt-like 1 (Drosophila) (gene)
LNK	lymphocyte-specific adaptor protein Lnk (gene)
LOH	loss of heterozygosity
LRRK2	leucine-rich repeat kinase 2 (gene)
Mb	Megabase
MDM2	MDM2 oncogene, E3 ubiquitin protein ligase (gene)
MDM4	Mdm4 p53 binding protein homolog (mouse) (gene)
MDS	myelodysplastic syndromes
min	minute
MPL	myeloproliferative leukemia virus oncogene (gene)
MPN	myeloproliferative neoplasms
mRNA	messenger ribonucleic acid
MYC	v-myc myelocytomatosis viral oncogene homolog (avian) (gene)

NF1	neurofibromin 1 (gene)
NFE2	nuclear factor (erythroid-derived 2), 45kDa (gene)
NPM1	nucleophosmin (nucleolar phosphoprotein B23, numatrin) (gene)
NR3C1	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor) (gene)
OR	odds ratio
PCR	polymerase chain reaction
PD	Parkinson's disease
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide (gene)
PDGFRB	platelet-derived growth factor receptor, beta polypeptide (gene)
PMF	primary myelofibrosis
PRC2	polycomb repressive complex 2
PV	polycythemia vera
RB1	retinoblastoma 1 (gene)
RBBP6	retinoblastoma binding protein 6 (gene)
RING	really interesting new gene
RNA	ribonucleic acid
RNA-seq	RNA sequencing
RUNX1	runt-related transcription factor 1 (gene)
sAML	secondary acute myeloid leukemia
sec	second
SEGEX	Segregation Exclusion analysis
SETBP1	SET binding protein 1 (gene)
SF1	splicing factor 1 (gene)
SF3A1	splicing factor 3a, subunit 1, 120kDa (gene)
SF3B1	splicing factor 3b, subunit 1, 155kDa (gene)
SGK2	serum/glucocorticoid regulated kinase 2 (gene)
SH2B3	SH2B adaptor protein 3 (gene)
sMF	secondary myelofibrosis
SNP	single nucleotide polymorphism
SNV	single nucleotide variant
SRSF2	serine/arginine-rich splicing factor 2 (gene)
STAT	signal transducers and activators of transcription (genes)
TERC	telomerase RNA component

TERT	telomerase reverse transcriptase
TET2	ten-eleven translocation oncogene family member 2 (gene)
THPO	thrombopoietin (gene)
TP53	tumor protein p53 (gene)
U2AF1	U2 small nuclear RNA auxiliary factor 1 (gene)
UPD	uniparental disomy
VHL	von Hippel-Lindau tumor suppressor (gene)
WHO	World Health Organization
XPD	xeroderma pigmentosum D (gene)
Znf	zinc finger domain
ZRSR2	zinc finger (CCCH type), RNA-binding motif and serine/arginine rich 2 (gene)

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