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%

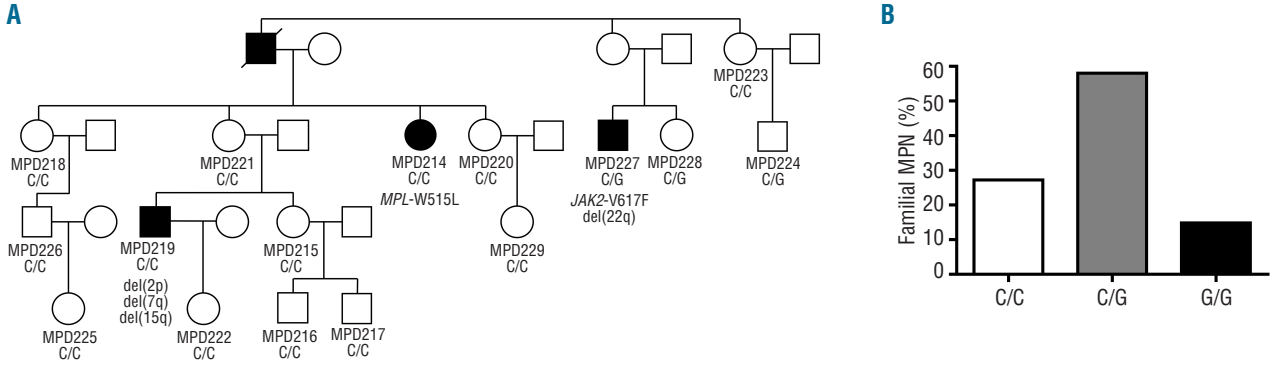

Figure 1. The *rs10974944* genotype and genetic heterogeneity in familial MPN. (A) Pedigree showing a MPN family with three affected members carrying distinct genetic aberrations. Family members with manifest MPN are shown as filled symbols with females represented as circles and males as squares. Patients 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.

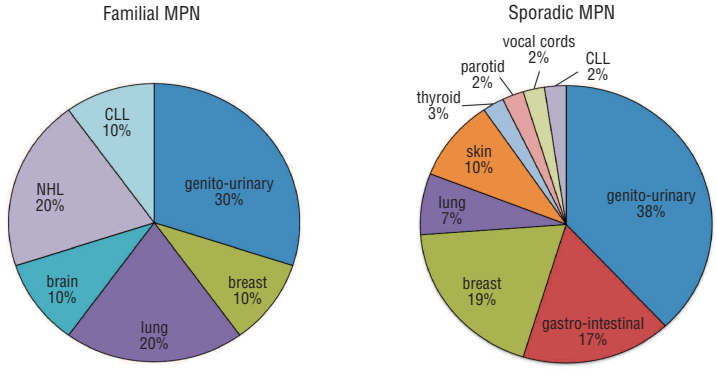

Figure 2. Malignancies in patients with familial or sporadic MPN. Pie charts represent the frequencies of malignant disorders in patients with familial or sporadic MPN. Type of cancer and frequencies in percentages are given for both groups. Lung and brain cancer as well as non-Hodgkin's lymphomas (NHL) 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

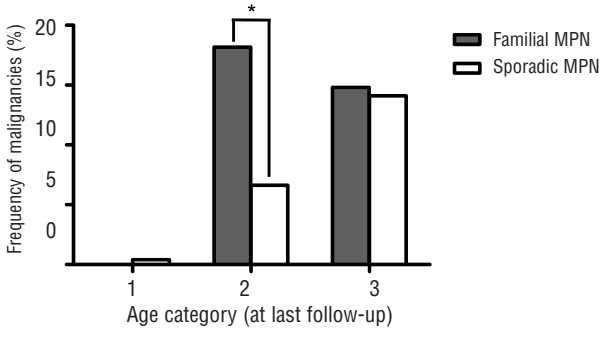

Figure 3. Frequency of malignancies in patients with familial and sporadic MPN according to age at last follow-up. Patients were grouped into three age categories (<50 years, 50-70 years, >70 years) according to age at last follow-up. Gray bars represent familial and white bars sporadic MPN cases. There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

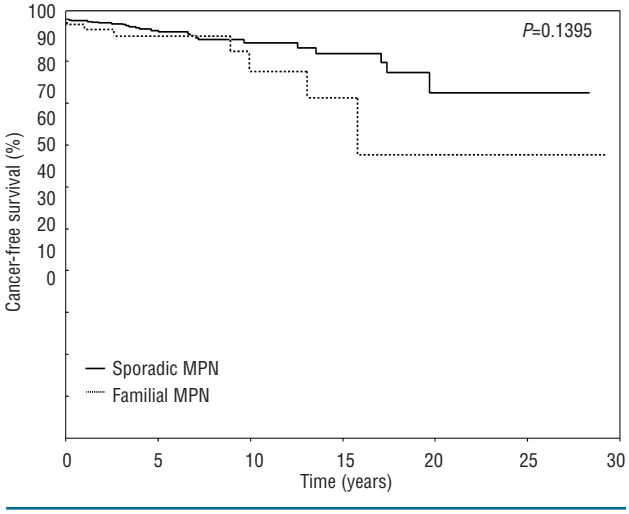

Figure 4. Cancer-free survival after the diagnosis of MPN in patients with familial or sporadic MPN. Kaplan-Meier estimation of cancer-free survival in familial MPN (dashed line) and sporadic MPN (black line). The x-axis shows time after MPN diagnosis in years and the y-axis represents the percentage 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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