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Abstract: Polycythemia vera, essential thrombocythemia and primary myelofibrosis are myeloproliferative neoplasms, characterized in a majority of cases by a unique somatic point mutation, JAK2 V617F. Recently, it was shown that JAK2 V617F occurs more frequently on a specific JAK2 haplotype, named JAK2 46/1. We genotyped 149 myeloproliferative neoplasms patients (69 had polycythemia vera, 65 had essential thrombocythemia and 15 had primary myelofibrosis) with a known JAK2 V617F mutational status and 150 controls for the JAK2 rs10974944 (C/G) SNP (single nucleotide hism), i h the G allele tags the 46/1 haplotype. We found tha the rs10974944 GG/CG genotypes were significantly enriched in patients compared to cols (< 0.0001). After stratifying for the JAK2 V617F mutational status and for the mutant allele burden, we demonstrated that GG/CG genotypes were significantly more frequent in V617F positive compared to V617F negative patients (p = 0.001), but not in V617F negative patients compared to controls (p = 0.29). Similarly, the GG/CG genotypes were significantly enriched in V617F positive patients with a mutant allele burden >50% compared to those with a mutant allele burden <50% (p = 0.0006). Our results indicate that the G allele, part of the JAK2 46/1 haplotype, contributes significantly to the oc of JAK2 617 - positive myeloproliferative neoplasms. Moreover, e JAK2 46/1 haplotype seems to be associated with mutant allele burden >50% in JAK2 617F - sitive myeloproliferative neoplasms patients.

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The G allele of the *JAK2* rs10974944 SNP, part of *JAK2* 46/1 haplotype, is strongly associated with *JAK2* V617F - positive myeloproliferative neoplasms

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Abstract

Polycythemia vera, essential thrombocythemia and primary myelofibrosis are myeloproliferative neoplasms, characterized in a majority of cases by a unique somatic point mutation, *JAK2* V617F. Recently, it was shown that *JAK2* V617F occurs more frequently on a specific *JAK2* haplotype, named *JAK2* 46/1. We genotyped 149 myeloproliferative neoplasms patients (69 had polycythemia vera, 65 had essential thrombocythemia and 15 had primary myelofibrosis) with a known *JAK2* V617F mutational status and 150 controls for the *JAK2* rs10974944 (C/G) SNP (single nucleotide polymorphism), in which the G allele tags the 46/1 haplotype. We found that the rs10974944 GG/CG genotypes were significantly enriched in patients compared to controls ($p < 0.0001$). After stratifying for the *JAK2* V617F mutational status and for the mutant allele burden, we demonstrated that GG/CG genotypes were significantly more frequent in V617F positive compared to V617F negative patients ($p = 0.001$), but not in V617F negative patients compared to controls ($p = 0.29$). Similarly, the GG/CG genotypes were significantly enriched in V617F positive patients with a mutant allele burden >50% compared to those with a mutant allele burden <50% ($p = 0.0006$). Our results indicate that the G allele, part of the *JAK2* 46/1 haplotype, contributes significantly to the occurrence of *JAK2* V617F - positive myeloproliferative neoplasms. Moreover, *JAK2* 46/1 seems to be associated with mutant allele burden >50% in *JAK2* V617F - positive myeloproliferative neoplasms patients.

Keywords: myeloproliferative neoplasms, *JAK2* V617F, *JAK2* 46/1 haplotype, *JAK2* rs10974944 SNP

Introduction

Myeloproliferative neoplasms (MPN) represent a heterogeneous group of hematological malignancies, characterized by clonal hematopoiesis and increased number of mostly mature peripheral blood elements of myeloid origin [1]. The most common BCR-ABL negative MPN are polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). Even though William Dameshek pointed out some common features of these 3 entities, including them in the same group of "myeloproliferative disorders" more than 50 years ago, [2] their molecular common point remained obscure until 2005, when several independent research groups reported that the majority of PV patients and about half of ET and PMF cases harbor a unique somatic mutation in the gene coding for Janus-kinase 2 (JAK2), a pivotal component of the JAK-STAT signalling pathway, essential for the differentiation and survival of the myeloid lineage [3 – 6]. This point mutation, leading to a substitution Val-to-Phe in position 617 of the JAK2 molecule, is highly specific to MPN, being designated a major diagnosis criterion for PV, ET and PMF according to the latest World Health Organization classification of MPN [7]. Although the discovery of the *JAK2* V617F mutation represents a major breakthrough in understanding the molecular pathogenesis of PV, TE and PMF, concerns have been raised regarding the mechanisms leading to the acquisition of this mutation. While some studies have shown that pre-*JAK2* events, such as chromosome 20q deletion, characterize at least part of the MPN patients [8], other focused on the presence of host-modifying genetic factors [9] or concomitant mutations in other genes, such as *TET2*, *ASXL1* or *MPL*, that could modulate the MPN phenotype

[10 – 13]. More recently, several independent groups reported that a particular *JAK2* haplotype, designated "46/1" or "GGCC", is strongly associated with the development of *JAK2* V617F - positive MPN [14 – 16], or with MPN development, regardless of the V617F mutational status [17, 18].

In the present study, we attempted to analyze whether the G allele of the *JAK2* rs10974944 SNP, which is in complete linkage disequilibrium with the 46/1 haplotype [14, 15] is associated with the predisposition to MPN and especially with the development of *JAK2* V617F - positive MPN. Moreover, we aimed to test the hypothesis of a possible relationship between *JAK2* rs10974944 SNP and mutant allele burden in *JAK2* V617F - positive MPN patients.

Materials and methods

Patients and controls

One hundred and forty-nine consecutive MPN patients (69 PV, 65 ET and 15 PMF), diagnosed between 1985 and 2009, were included in the study, after carefully revising their diagnosis according to the latest World Health Organization classification of MPN. The control group comprised 150 unrelated local volunteers, which were referred for routine blood workup at the Department of Hematology, "Ion Chiricuta" Cancer Institute, Cluj-Napoca, Romania and satisfied the following conditions: age above 60 years and absence of hematological malignancy. Written consent regarding genetic testing was obtained from all patients and controls. All experiments were performed according to the World Medical Association Declaration of Helsinki. This study was

reviewed and approved by the Ethics Committee of the "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania.

Molecular analysis for JAK2 V617F mutation and JAK2 rs10974944 SNP

Genomic DNA was obtained from peripheral leukocytes, using a commercially available kit (Wizard Genomic DNA Purification Kit, Promega, WI, USA). The *JAK2* V617F mutational status was assessed semiquantitatively in all patients by a tetra-primer PCR assay, as previously described [1].

In order to obtain a rapid screening method for the putative *JAK2* V617F – predisposing haplotype, we developed a PCR-RFLP (Polymerase Chain Reaction – Restriction Fragments Length Polymorphism) assay for analyzing the *JAK2* rs10974944 SNP, which belongs to this haplotype. We described in detail the method elsewhere [19]. The PCR-RFLP assay was validated by DNA sequencing.

As it has been previously described, the *JAK2* V617F mutation tends to occur more frequently in *cis* with the G allele of the rs10974944 [16]; thus, a CG heterozygous peripheral blood sample would be erroneously labelled as GG homozygous if it contained almost exclusively the *JAK2* V617F mutation. In order to avoid this potential misinterpretation, genomic DNA was obtained from buccal cells from 22 MPN patients (20 PV patients and 2 ET patients), which harboured *JAK2* V617F in a proportion close to 100% and seemed homozygous for the G allele of the rs10974944 SNP, when their peripheral blood DNA was analyzed. These patients were genotyped once again for the *JAK2* rs10974944 SNP using their buccal cell DNA, which provided their correct constitutional genotype, which was proven to be CG in 14 of them (13 PV patients and 1

PV patient); these patients were subsequently included in the CG group for the statistical analysis.

Statistical analysis

The distribution of qualitative variables between different groups was compared by the Fisher's exact test. Odds-ratio (OR) and 95% confidence interval (CI) were also calculated for each comparison performed. The *p* values were two-tailed and the results were considered statistically significant if the *p* value was less than 0.05. The statistical analysis was performed using the GraphPad InStat software, version 3 (GraphPad, San Diego, CA, USA).

Results

The JAK2 V617F mutation and mutant allele burden distribution in MPN patients

Overall, 107 MPN patients (71.8%) were *JAK2* V617F-positive. When analyzing each MPN entity in part, it was found that 61 PV patients (88.4%), 37 ET patients (57%) and 9 PMF patients (60%) were *JAK2* V617F-positive. Of the *JAK2* V617F positive patients, 69 (64.5%) had an allele burden >50%, of which 49 (80.3%) were PV, 12 (32.4%) were ET and 8 (88.9%) were PMF patients.

The JAK2 rs10974944 SNP distribution in patients and controls

Table 1 shows in detail the distribution of *JAK2* rs10974944 SNP genotypes and alleles in patients, stratified by *JAK2* V617F mutational status and mutant allele burden,

and controls. The GG/CG genotypes were significantly enriched in the MPN group compared to controls ($p < 0.0001$; OR = 3.4; 95% CI = 2.1 – 5.5). Similarly, the G allele was found at a significantly higher frequency in patients than in controls ($p < 0.0001$; OR = 2.3; 95% CI = 1.7 -3.3). Moreover, we noticed a significant excess of GG/CG genotypes in PV compared to ET patients ($p = 0.0001$; OR = 5.4; 95% CI = 2.2 – 13.2), which was also true for the G allele frequency ($p = 0.0004$; OR = 2.5; 95% CI = 1.5 – 4), which could be due to the higher incidence of the *JAK2* V617F mutation in PV compared to ET.

The JAK2 rs10974944 SNP distribution in MPN patients stratified by the JAK2 V617F mutational status and by the mutant allele burden

Eighty seven out of one hundred and seven (81.3%) MPN patients, positive for *JAK2* V617F mutation, carried at least one copy of the G allele, compared to 23 out 42 (54.8%) patients, negative for *JAK2* V617F mutation ($p = 0.001$; OR = 3.6; 95% CI = 1.7 – 7.8). Also, the G allele had a significantly increased frequency in V617F - positive patients compared to V617F - negative patients ($p = 0.006$; OR = 2.1; 95% CI = 1.2 – 3.5). Similarly, the CG/GG genotypes, as well as the G allele, were significantly more frequent in *JAK2* V617F - positive MPN patients than in controls ($p<0.0001$; OR = 5.2; 95% CI = 2.9 – 9.4 for GG/CG genotypes and $p < 0.0001$; OR = 2.8; 95% CI = 2 – 4.1 for the G allele). Meanwhile, when comparing the rs10974944 GG/CG genotypes distribution and G allele frequency between *JAK2* V617F negative MPN patients and controls, no significant differences were observed ($p = 0.29$; OR = 1.5; 95% CI = 0.7 – 2.9 for GG/CG genotypes and $p = 0.27$; OR = 1.4; 95% CI = 0.8 – 2.3 for the G allele).

Sixty three out of sixty nine (91.3%) V617F positive MPN patients with a mutant allele burden >50% harboured either the GG or CG genotype compared to 24 out 38 (63.2%) MPN patients with a mutant allele burden <50% (p = 0.0006; OR = 6.1; 95% CI = 2.1 – 17.8). Also, the G allele had a significantly increased frequency in patients with a mutant allele burden >50% than in those with <50% (p = 0.001; OR = 2.7; 95% CI = 1.5 – 4.7). Similarly, the GG/CG genotypes, as well as the G allele, were significantly enriched in MPN patients with a mutant allele burden >50% compared to controls (p < 0.0001; OR = 12.7; 95% CI = 5.2 – 31.1 for GG/CG genotypes and p < 0.0001; OR = 4; 95% CI = 2.6 - 6.1 for the G allele). However, neither the GG/CG genotypes, nor the G allele were significantly enriched in patients with a mutant allele burden <50% compared to controls (p = 0.06; OR = 2.1; 95% CI = 1 – 4.3 for GG/CG genotypes and p = 0.15; OR = 1.5; 95% CI = 0.9 – 2.6 for the G allele). Table 2 analyses the relationship between the *JAK2* V617F mutation and the rs10974944 SNP, while table 3 presents the relationship between *JAK2* mutant allele burden and the rs10974944 SNP.

Discussion

The recent studies have shown an association between *JAK2* V617F - positive MPN and the *JAK2* 46/1 haplotype [14 – 18], our data confirming these results. However, we could not confirm the previous reports that the *JAK2* 46/1 haplotype also increases susceptibility to *JAK2* V617F negative ET [17] and PMF [18]. The analysis based on *JAK2* V617F stratification revealed that only the *JAK2* V617F - positive MPN cases were significantly associated with the rs10974944 SNP, either in heterozygous or

homozygous state. As these discrepancies could be related to the differences regarding the size of the analyzed patients cohorts, larger studies would probably clarify the contribution of the *JAK2* 46/1 haplotype to the V617F negative MPN. The association between the 46/1 haplotype and both the *JAK2* V617F - positive and negative MPN cases would support the hypothesis that this haplotype acts as a functional variant influencing the clonal expansion and disease manifestation [17].

The "GGCC" haplotype seems to confer susceptibility not only to the *JAK2* V617F mutation, but to *JAK2* exon 12 mutations as well. Recently, it was observed that the rs10974944, which tags the "GGCC" haplotype, is significantly enriched in PV patients positive for *JAK2* exon 12 point mutations or deletions, which characterize a minority of PV patients negative for the V617F mutation [20]. Based on these observations, it is presumable that a specific *JAK2* haplotype induces a hypermutable milieu within the *JAK2* gene. This hypothesis is supported by the findings that the *JAK2* V617F mutation occurred preferentially in *cis* with the C allele of *JAK2* rs12343867 SNP (part of the 46/1 haplotype) [14, 15] and the G allele of the *JAK2* rs10974944 SNP [16].

We also attempted to assess whether the *JAK2* 46/1 haplotype influences the expansion of the *JAK2* V617F mutant clone and indeed we found that the G allele, either in heterozygous or homozygous state was significantly enriched in patients with >50% V617F mutant allele compared to patients with <50% mutant allele; these data are in agreement with two recent reports, which have shown that the *JAK2* 46/1 haplotype was significantly enriched in ET patients with mutant allele burden higher than 10% compared to those who had less than 10% [17] and PMF patients with mutant allele burden higher than 20% compared to those who had less than 20% [18]. These findings

suggest that the *JAK2* 46/1 could play a role not only in the acquisition of *JAK2* V617F mutation, but also in the survival and expansion of the V617F mutant clone, probably by conferring a selective advantage. These findings could also suggest an influence that the *JAK2* 46/1 haplotype might have in the mitotic recombination of chromosome 9p, a common mechanism by which the *JAK2* V617F mutation becomes homozygous [3].

In conclusion, we demonstrated that the G allele of the *JAK2* rs10974944 SNP is a significant risk factor for *JAK2* V617F - positive MPN. Our results would support the hypothesis of a hypermutagenesis milieu within the *JAK2* gene, given that we did not observe the association of the G allele with *JAK2* V617F - negative MPN; however, a possible functional consequence of the *JAK2* 46/1 haplotype influencing the JAK-STAT signalling pathway cannot be ruled out. Even though the mechanisms behind *JAK2* V617F acquisition are not yet well understood, these recent findings could provide novel insights in understanding the molecular pathophysiology of MPN. Future studies will probably identify other germline variants involved in the pathogenesis of MPN.

Autorship and conflict of interest disclosure

APT designed research, performed genetic analysis, analyzed data and wrote the manuscript. AC designed research, collected the samples and patients data, analyzed data and wrote the manuscript. LP, LU and DD collected the samples and patients data. MSM and IVP performed genetic analysis. RAP designed research and analyzed data.
The authors reported no potential conflict of interest.

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Table 1. *JAK2* rs10974944 SNP genotype and allele frequency in MPN patients and controls

rs10974944 genotype/ allele	MPN overall (N = 149)				Controls (N = 150)
	V617F positive (N = 107)		V617F negative (N = 42)	Total	
	allele burden >50% (N = 69)	allele burden <50% (N = 38)			
Genotype frequency; n (%)					
CC	6 (8.7)	14 (36.8)	19 (45.2)	39 (26.2)	82 (54.7)
CG	46 (66.7)	22 (57.9)	19 (45.2)	87 (58.4)	59 (39.3)
GG	17 (24.6)	2 (5.3)	4 (9.6)	23 (15.4)	9 (6)
Allele frequency; n (%)					
C allele	58 (42)	50 (66)	57 (67.8)	165 (55.4)	223 (74.3)
G allele	80 (58)	26 (34)	27 (32.2)	133 (44.6)	77 (25.7)

Table 2. Correlation between *JAK2* rs10974944 SNP genotypes/ alleles stratified by *JAK2* V617F mutational status

rs 10974944 genotype/ allele	MPN overall vs controls		*JAK2* V617F positive vs *JAK2* V617F negative MPN		*JAK2* V617F positive MPN vs control group		*JAK2* V617F negative MPN vs control group	
	OR [95% CI]	*p* value	OR [95% CI]	*p* value	OR [95% CI]	*p* value	OR [95% CI]	*p* value
GG vs CG/CC	2.9 [1.3-6.4]	0.008	2.1 [0.7-6.4]	0.3	3.4 [1.5-7.8]	0.004	1.7 [0.5-5.7]	0.48
GG vs CC	5.4 [2.3-2.7]	<0.0001	4.5 [1.3-15.7]	0.01	8.7 [3.4-22]	<0.0001	1.9 [0.5-6.9]	0.29
CG vs CC	3.2 [1.9-5.1]	<0.0001	3.4 [1.5-7.6]	0.003	4.7 [2.6-8.6]	<0.0001	1.4 [0.7-2.9]	0.46
GG/CG vs CC	3.4 [2.1-5.5]	<0.0001	3.6 [1.7-7.8]	0.001	5.2 [2.9-9.4]	<0.0001	1.5 [0.7-2.9]	0.29
G allele vs C allele	2.3 [1.7-3.3]	<0.0001	2.1 [1.2-3.5]	0.006	2.8 [2-4.1]	<0.0001	1.4 [0.8-2.3]	0.27

Table 3. Correlation between *JAK2* rs10974944 SNP genotypes/ alleles stratified by *JAK2* V617F allele burden

rs 10974944 genotype/ allele	V617F allele burden higher than 50% vs allele burden lower than 50%		V617F allele burden higher than 50% vs control group		V617F allele burden lower than 50% vs control group	
	OR [95% CI]	*p* value	OR [95% CI]	*p* value	OR [95% CI]	*p* value
GG vs CG/CC	5.9 [1.3-27]	0.01	5.1 [2.1-12.2]	0.0002	0.9 [0.2-4.2]	1
GG vs CC	19.8 [3.4-114]	0.0002	25.8 [8.1-82.2]	<0.0001	1.3 [0.3-46.7]	0.66
CG vs CC	4.9 [1.7-14.4]	0.004	10.7 [4.3-26.6]	<0.0001	2.2 [1-4.6]	0.04
GG/CG vs CC	6.1 [2.1-17.8]	0.0006	12.7 [5.2-31.1]	<0.0001	2.1 [1-4.3]	0.06
G allele vs C allele	2.7 [1.5-4.7]	0.001	4 [2.6-6.1]	<0.0001	1.5 [0.9-2.6]	0.15

***Conflict of interest**

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