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Chronic myeloproliferative neoplasms

The -2518 A/G polymorphism of the monocyte chemoattractant protein-1 as a candidate genetic predisposition factor for secondary myelofibrosis and biomarker of disease severity

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Philadelphia-negative myeloproliferative neoplasms (MPN), namely polycythemia vera (PV), essential thrombocythemia (ET) and myelofibrosis (MF), are closely-related stem cell disorders, characterized by abnormal proliferation and differentiation of hematopoietic progenitors [1, 2]. Transitions between disease entities are common, shaping a "biological continuum" from an early stage with a relatively milder phenotype (PV and ET) toward an advanced phase, termed secondary myelofibrosis (sMF) [3]. Similarly, pre-fibrotic and overt primary myelofibrosis (pre-PMF and overt-PMF, respectively), according to the 2016 WHO criteria [4] have been shown to be aligned along a phenotypic gradient of severity [5]. Although different biomarkers have been associated with MPN thrombotic comorbidities [6, 7], no known parameters for predicting whether PV or ET will advance to sMF or for establishing a timeline for the progression of pre-PMF into overt disease currently exist.

Chronic inflammation plays a pivotal role in MPN pathogenesis, triggering neoplastic transformation and catalyzing clonal evolution toward end-stage disease. Indeed, MPN cells release a plethora of pro-inflammatory products, which in turn elicits genomic instability and drive clonal myeloproliferation [3, 8]. It has been demonstrated that: (i) MF patients display higher circulating levels of several pro-

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Table 1 Genotypic and allelic frequencies of the -2518 A/G SNP of MCP-1 in overall MPN population, PV, ET, MF patients and CTRL

	Genotypic frequencies				Allelic frequencies	
	A/A *n* (%)	A/G *n* (%)	G/G *n* (%)	A/G + G/G *n* (%)	A allele	G allele
MPN (*n* 177)	94 (53.1)	74 (41.8)	9 (5.1)	83 (46.9)	0.740	0.260
PV (*n* 44)	26 (59.1)	15 (34.1)	3 (6.8)	18 (40.9)	0.761	0.239
ET (*n* 65)	31 (47.7)	33 (50.8)	1 (1.5)	34 (52.3)	0.731	0.269
MF (*n* 68)	37 (54.4)	26 (38.2)	5 (7.4)	31 (45.6)	0.735	0.257
CTRL (*n* 149)	90 (60.4)	53 (35.6)	6 (4.0)	59 (39.6)	0.782	0.218

P n.s. in all comparisons

inflammatory cytokines as compared to other chronic myeloproliferative disorders as well as to healthy subjects [9], with IL-8, IL-2R, IL-12 and IL-15 levels independently holding prognostic value [10]; (ii) MCP-1 (monocyte chemoattractant protein-1, also known as CCL2), soluble IL-2R and IL-15 levels cluster with splenomegaly [11]; (iii) MCP-1 levels correlate with lower anemia response to pomalidomide [11].

MCP-1 is the main chemotactic factor for monocyte migration in sites of inflammation and contributes to organ fibrotic changes [12]. MCP-1 expression levels are highly variable among individuals, potentially contributing to differential susceptibility to various inflammatory conditions [13]. An A to G single-nucleotide polymorphism (SNP) in MCP-1 enhancer region (rs1024611, originally designated as –2518 G or –2578 G) was found to be responsible for higher levels of MCP-1 production by monocytes upon inflammatory noxa [14], and has been associated to several chronic inflammatory conditions such as autoimmune disorders, atherosclerosis and chronic infectious diseases [15]. In the present study, we investigated whether the -2518 A/G SNP of MCP-1 is a potential indicator of MPN susceptibility and/or disease phenotype.

After approval by the local ethical committee (prot. *n* 27182) and written informed consent, *n* 177 Caucasian MPN patients were recruited, of which *n* 44 PV, *n* 65 ET, *n* 68 MF (*n* 45 PMF and *n* 23 sMF). For PMF patients, histopathology, clinical and laboratory data were reviewed and diagnoses attributed to pre-PMF (*n* 12) or overt-PMF (*n* 33) according to the revised 2016 WHO criteria [4]. DNA was extracted by PureLink® Genomic DNA Kit (Invitrogen) from 200 µl of whole blood and from buccal mucosa cells following manufacturer's instructions. DNA from 149 age-matched and sex-matched Caucasian healthy subjects (CTRL) was provided by the Unit of Medical Genetics, University Hospital of Parma. Patients and CTRL genotyping was performed by TaqMan® Predesigned SNP Genotyping Assays (Applied Biosystems). Patients' data were retrospectively analyzed from cataloged hospital records.

For statistical analysis, numerical variables were summarized by their median and range, and categorical variables by count and relative frequency (percentage). Differences in the distribution of continuous variables were calculated by Mann–Whitney/Kruskal–Wallis tests, while categorical variable comparison were established by χ^2/Fisher exact test. A P value <0.05 was considered statistically significant. Analysis was performed with dedicated software (Epi Info 7.2.1.0; CDC, Atlanta, GA, USA or StatView 5.0; SAS Institute Inc, Cary, NC, USA).

Case and control groups were aligned for age and gender distribution. Clinical and biological characteristics of MPN patients and CTRL are summarized in Supplemental Table 1.

Genotypic and allelic frequencies of the MCP-1 -2518 A/G SNP in MPN and CTRL are reported in Table 1. Genotypic frequencies were in Hardy–Weinberg equilibrium both in the MPN patients and CTRL ($P > 0.05$). No statistical differences were found by comparing genotypic and allelic frequencies of overall MPN, PV, ET and MF patients vs. CTRL, as well as between single disease entities.

Focusing on MF, which is the MPN variant characterized by the highest inflammation burden [16], we evaluated whether polymorphic genotypes could be associated to specific disease subtype(s) (based on the 2016 WHO criteria) or to disease phenotype aggressiveness based on the hematologic characteristics at the time of diagnosis (Table 2).

We found that the subjects carrying either a heterozygous or homozygous genotype for the -2518 A/G SNP (A/G + G/G) were significantly more frequent in sMF vs. PMF (17/23, 73.9% vs. 14/45, 31.1%, respectively, $P = 0.0008$, Table 2). Additionally, sMF was significantly more frequent in A/G + G/G patients than either pre-PMF (1/12, 8.3%, $P = 0.0002$) or overt-PMF (13/33, 39.4%, $P = 0.011$). Notably, the number of A/G + G/G subjects was also significantly higher in sMF as compared to CTRL ($P = 0.022$) (Table 2). The observation that sMF is enriched in allele-G carriers is consistent with the concept of myelofibrosis as a burn-out phase of a long process that starts with ET/PV and advances

Table 2 Genotype–phenotype correlations in MF patients

	No. of cases	A/A	A/G + G/G	*P* [O.R., 95% C.I.]
Disease type				
PMF, *n* (%)	45	31 (68.9)	14 (31.1)	***P* = 0.0008 vs. sMF [6.23; 2.04–19.32]**
Pre-PMF, *n* (%)	12	11 (91.7)	1 (8.3)	***P* = 0.0002 vs. sMF [31.17; 3.29–295.35]** $P = 0.07$ vs. overtPMF
Overt-PMF, *n* (%)	33	20 (60.6)	13 (39.4)	***P* = 0.011 vs. sMF [4.36; 1.36–13.95]**
sMF *n* (%)	23	6 (26.1)	17 (73.9)	***P* = 0.022 vs. CTRL [3.07; 1.14–8.32]**
Age				
Median (range), years	68	69.0 (29–84)	70.0 (30–86)	$P = 0.61$
>65 years, *n* (%)	46	25 (54.4)	21 (45.6)	$P = 0.99$
Gender				
Male, *n* (%)	41	21 (51.2)	20 (48.8)	$P = 0.51$
Female, *n* (%)	27	16 (59.3)	11 (40.7)	
IPSS				
Low/intermediate-1, *n* (%)	42	28 (66.7)	14 (33.3)	***P* = 0.0078 [4.29; 1.42–12.91]**
Intermediate-2/high, *n* (%)	22	7 (31.8)	15 (68.2)	
Hemoglobin				
Median (range), g/L	62	12.7 (5–15.9)	11.7 (7.3–15.5)	$P = 0.062$
<100 g/L, *n* (%)	13	4 (30.8)	9 (69.2)	***P* = 0.036 [3.89; 1.04–14.41]**
WBC				
Median (range), $x10^9$/L	62	9.2 (3.9–57.8)	12.1 (2.9–57.0)	$P = 0.78$
$<4 \times 10^9$/ L or $>25 \times 10^9$/L, *n* (%)	7	3 (42.9)	4 (57.1%)	$P = 0.44$
Platelets				
Median (range), $\times10^9$/L	60	560 (99–1322)	376 (69–984)	$P = 0.10$
LDH				
Median (range), U/L	59	622 (205–1620)	751 (343–1580)	$P = 0.22$
>Normal range, *n* (%)	47	26 (55.3)	21 (44.7)	$P = 0.85$
Constitutional symptoms				
Yes, *n* (%)	47	7 (41.8)	10 (58.8)	$P = 0.19$
No, *n* (%)	17	28 (59.6)	19 (40.4)	
Circulating blasts				
<1%, *n* (%)	53	33 (62.3)	20 (37.4)	***P* = 0.014 [6.6; 1.27–34.23]**
≥1%, *n* (%)	10	2 (20.0)	8 (80.0)	
Grading of fibrosis				
0–I, *n* (%)	29	20 (69.0)	9 (31.0)	***P* = 0.048 [2.78; 0.99–7.43]**
≥II, *n* (%)	36	16 (44.4)	20 (55.6)	
Spleen (long. Ø by US) median (range), cm	68	14.0 (7.5–30)	17.0 (10–30)	$P = 0.1$
*JAK2*V617F mutation				
Positive, *n* (%)	40	21 (52.5)	19 (47.5)	$P = 0.44$
Negative, *n* (%)	19	12 (63.2)	7 (36.8)	
Major thrombotic events				
Yes, *n* (%)	22	11 (50.0)	11 (50.0)	$P = 0.55$
No, *n* (%)	45	26 (57.8)	19 (42.2)	

Statistically significant associations are highlighted in bold, and relative Odds ratio (O.R.) and 95% Confidence Interval (C.I.) are reported

Age, IPSS risk category, leukocytes, hemoglobin, platelets, presence of blasts, LDH constitutional symptoms and spleen size refer to the time of diagnosis. "No. of cases" (second column) refers to: (i) for non-continuous variables, the no. of patients presenting the indicated parameter (i.e., no. of JAK2V617 positive and negative patients); (ii) for continuous variables, the no. of patients evaluated for that parameter (i.e., age at the time of diagnosis).

toward a more progressive disease state, characterized by higher inflammation burden [3, 16]

Genotype–phenotype correlation studies in MF patients revealed a higher frequency of allele-G carriers (A/G + G/G) in: (i) intermediate-2/high vs. low/intermediate-1 IPSS risk group (15/22, 68.2% vs. 14/42, 33.3%, respectively, $P = 0.0078$), (ii) patients with lower (Hb < 100 g/L) vs. higher (Hb ≥ 100 g/dL) hemoglobin levels (9/13, 69.2% vs. 18/49, 36.7%, $P = 0.036$, (iii) patients with (≥1%) vs. patients without (<1%) circulating blasts (8/10, 80%, vs. 20/53, 37.4%, $P = 0.014$), (iv) patients with higher (≥II) vs. lower (0–I) grading of bone marrow fibrosis (20/36, 55.6% vs. 9/29, 31.0%, $P = 0.048$) (Table 2).

No associations with age, gender, white blood cell and platelet count, LDH levels, presence of constitutional symptoms, spleen size, JAK2V617F mutation, and history of major thrombotic events were found (Table 2).

Finally, to evaluate whether the MCP-1 -2518 A/G SNP is inherited or acquired by hematopoietic stem cells, we tested the SNP in non-clonal cells of 14 MPN patients (10 MF, 3 ET and 1 PV) harboring the G allele, as assessed by whole blood genotyping. The analysis of buccal mucosal cells revealed that all individuals were germline carriers of the polymorphism.

In conclusion, our data suggest that the -2518 A/G SNP of MCP-1 could represent a host genetic predisposition factor for sMF and may serve as a biomarker of disease severity in MF, as implied by its association with higher IPSS, peripheral blasts, lower hemoglobin and higher grading of bone marrow fibrosis. In particular, the association of the SNP with higher grading of bone marrow fibrosis as well as with severe anemia is consistent with the well-defined pro-fibrotic role of this chemokine [12] and the previously described observation that MCP-1 levels correlates with poor anemia response [11]. We speculate that this SNP, after prospective validation studies, may configure as a genetic biomarker identifying ET and PV patients who more likely will progress toward a spent phase.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Acute myeloid leukemia

Minimal residual disease (MRD) monitoring and mutational landscape in AML with *RUNX1-RUNX1T1*: a study on 134 patients

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The cure rate in AML depends on patient's age and performance status, cytogenetics, early blast clearance and sustainable first complete remission. Investigation of minimal residual disease (MRD) is possible by multiparameter-flow cytometry or molecular techniques. Recent findings have further depicted a broad spectrum of molecular markers in AML in 99% of patients [1]. This broadens the set of targets for MRD assessment and will hopefully help to better individualize treatment strategies. MRD monitoring by qPCR is feasible in AML with *RUNX1-RUNX1T1* fusion. The absence of *RUNX1-RUNX1T1* transcripts is considered as complete molecular remission (CMR). Risk stratification according to MRD is possible and initial studies allocating MRD positive patients to allogeneic stem cell transplantation have been undertaken [2]. However, despite CMR about 10–30% of patients relapse [3, 4].

This analysis aims to understand the clinical use of PCR based MRD monitoring in AML with *RUNX1-RUNX1T1* fusion outside clinical trials. We specifically address chosen time points for measurements, choice of peripheral blood (PB) vs bone marrow (BM) as sample material for follow-up testing and evaluate the value of CMR as an absolute MRD negativity. In addition, we performed 63 gene panel sequencing to analyze recurrent mutations and their association to CMR and outcome.

Between 2005 and 2017 a total of 134 intensively treated AML patients with *RUNX1-RUNX1T1* fusion were diagnosed and followed at our laboratory (for characteristics see supplemental table 1). We analyzed 1081 individual samples (supplemental table 2) during that time. We applied absolute quantitative real-time PCR to measure *RUNX1-RUNX1T1/ABL1* ratios [5]. Complete molecular remission (CMR) was defined as qPCR ratio of 0 (sensitivity 0.001%) and negative nested PCR. Low MRD was assigned to patients with qPCR ratio of 0 but positive nested PCR and high MRD was assigned to all patients with a ratio above 0.

There was no pre-specified time point for MRD monitoring and the median time between two investigations of 2.9 months (range 0.5–61 months) reflects the everyday use of MRD in clinical practice. CMR was reached in 79 out of 134 patients (59%) after a median of 8 months (range 1–46 months). CMR was preferentially defined in BM and only 1 out of 79 patients had CMR detected in PB only. In total 15 out of 134 (11%) patients reached low MRD with a positive nested PCR, and 40 out of 134 (30%) reached high-level MRD (median lowest *RUNX1-RUNX1T1/ABL1* ratio of 0.022% (range 0.001–5.4%). Median relapse free survival (RFS) of patients with CMR was not reached (RFS at 2 years 82%; 95% CI, 75–92%) and significantly longer (both $p < 0.001$) than for low MRD and high MRD patients (16 months (range 5–65) and 13 months (range 3–45), respectively, not significant (n.s.), Fig. 1a). Overall survival rate at 5 years was 80% (95% CI, 66–88%) and 75% (95%

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