



Chronic myeloproliferative neoplasms

miR-146a rs2431697 identifies myeloproliferative neoplasm patients with higher secondary myelofibrosis progression risk

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Abstract

Myelofibrosis (MF) occurs as part of the natural history of polycythemia vera (PV) and essential thrombocythemia (ET), and remarkably shortens survival. Although *JAK2V617F* and *CALR* allele burden are the main transformation risk factors, inflammation plays a critical role by driving clonal expansion toward end-stage disease. NF-κB is a key mediator of inflammation-induced carcinogenesis. Here, we explored the involvement of miR-146a, a brake in NF-κB signaling, in MPN susceptibility and progression. rs2910164 and rs2431697, that affect miR-146a expression, were analyzed in 967 MPN (320 PV/333 ET/314 MF) patients and 600 controls. We found that rs2431697 TT genotype was associated with MF, particularly with post-PV/ET MF (HR = 1.5; $p < 0.05$). Among 232 PV/ET patients (follow-up time = 8.5 years), 18 (7.8%) progressed to MF, being MF-free-survival shorter for rs2431697 TT than CC + CT patients ($p = 0.01$). Multivariate analysis identified TT genotype as independent predictor of MF progression. In addition, TT (vs. CC + CT) patients showed increased plasma inflammatory cytokines. Finally, miR-146a^{-/-} mice showed significantly higher Stat3 activity with aging, parallel to the development of the MF-like phenotype. In conclusion, we demonstrated that rs2431697 TT genotype is an early predictor of MF progression independent of the *JAK2V617F* allele burden. Low levels of miR-146a contribute to the MF phenotype by increasing Stat3 signaling.

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Introduction

Transformation to secondary myelofibrosis (MF) occurs as part of the natural history of polycythemia vera (PV) and essential thrombocythemia (ET), which are the two most indolent Philadelphia-negative myeloproliferative neoplasms

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(MPNs). Once MF appears, survival is remarkably shortened [1]. Although not fully characterized, risk factors for progression to MF include the type of MPN (higher in PV than in ET); the time from diagnosis [1]; a *JAK2V617F* allele burden >50% in post-PV MF [2]; *CALR* mutations in post-ET MF [3]; and the presence of cooperating mutations in myeloid genes [4, 5]. Several lines of evidence, however, highlight the role of inflammation in the pathogenesis and progression of MPNs as a trigger and driver of clonal expansion from early stage disease to the myelofibrotic burn-out phase [6]. Importantly, the JAK/STAT signaling pathway, involving JAK1, JAK2, STAT3, and STAT5, plays a critical role in MPN pathogenesis by driving not only the malignant clone, but also the inflammatory process [7, 8]. Indeed, based on a single-cell profiling approach, a recent study demonstrated that JAK/STAT3-mediated cytokine production from both malignant and nonmalignant cells contributes to MPN pathogenesis and, most importantly, that *Stat3* deletion restricted to MPN cells in a mouse model does not reduce disease severity or cytokine production [7]. Moreover, although JAK inhibitors have provided a substantial clinical benefit, their disease-modifying activity is limited [9], underscoring the need to identify other pathogenic pathways to target.

NF- κ B has been proposed as a key mediator of inflammation-induced carcinogenesis [10, 11]. As in other malignancies, NF- κ B activation is detected in MF [12] and has been implicated in the abnormal release of TGF- β 1, a cytokine that potently stimulates fibroblasts to produce collagen and bone [13]. NF- κ B is regulated by microRNAs (miRNAs), small noncoding RNA molecules that regulate target gene expression [14, 15]. Specifically, miR-146a is a negative regulator of the NF- κ B pathway by inhibiting the expression of TNF receptor-associated factor 6 protein (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1) [16], two upstream signal transducers that are crucial for pro-inflammatory signaling. Under inflammatory stimulation, NF- κ B itself promotes the expression of cytokines, such as IL-6, IL-8 [16–18], IL-1 β [19], TGF- β 1 [13], MCP-1, IL12p70 [20], as well as miR-146a [21]. This miRNA acts as a negative feedback regulator to control and avoid excessive inflammatory signaling [21]. Interestingly, aging miR-146a-deficient mice (*miR-146a*^{-/-}) display exaggerated inflammatory responses to bacterial challenges and, over time, develop splenomegaly, myeloproliferation, extramedullary hematopoiesis [16], and bone marrow (BM) fibrosis [18], a MF-like phenotype. Human miR-146a expression levels are decreased in a miRNA array performed in granulocytes from primary MF patients [22], as well as in the BM of patients with 5q-syndrome [23]. Importantly, the levels of miR-146a are regulated by the presence of the functional polymorphisms (miR-SNPs) rs2431697 (~1 kb 5' of *MIR146A*) and rs2910164 (inside

the *MIR146A* gene) [24]. In particular, the rs2431697 TT genotype has been associated with lower levels of miR-146a than the CC genotype [24], thus behaving as a booster for the disease. Of note, these miR-SNPs have been linked to other inflammatory diseases, such as systemic lupus [24], thyroid papillary cancer [25], ischemic stroke [26], and cardiovascular complications in atrial fibrillation [27]. In this framework, we investigated whether the functional *MIR146A* miR-SNPs rs2431697 and rs2910164 were potential factors for MPN susceptibility. We hypothesized that *MIR146A* miR-SNPs, which regulate miR-146a expression levels, may result in a higher risk of developing MPN, specifically MF.

Materials and methods

Patients and controls

A total of 967 MPN patient samples were included in the study. The samples comprised 320 samples from patients with PV, 333 samples from patients with ET, and 314 samples from patients with MF (Supplementary Table 1). The samples from patients with PV and ET were collected from three Spanish institutions [Hospital Morales Meseguer (Murcia), Hospital del Mar (Barcelona), and Catalan Institute of Oncology (Barcelona)], whereas the MF cohort (180 primary MF patients and 134 post-PV/ET MF patients) were recruited from 13 Spanish institutions belonging to the Spanish group of Philadelphia-Negative Myeloproliferative Diseases (GEMFIN). The study was approved by the local ethics committees (Protocol number: MF-00015) and registered by the Spanish Drug and Health Products Agency (AEMPS) under the reference 26227/RG 67758. Informed consent for inclusion into the study was obtained in accordance with the requirements of local ethics committees.

In each case, the diagnosis was made according to the criteria used at the time of first observation and locally reviewed according to WHO criteria and the International Working Group-MRT for post-PV and post-ET MF [28, 29]. The study also included 600 healthy individuals, who were age- and sex-matched to the patients, as controls. All healthy subjects provided their written informed consent to participate in this study.

Genotyping

MIR146A miR-SNPs, namely, rs2431697 and rs2910164, were genotyped with DNA obtained from peripheral whole blood using Taqman® SNP Genotyping Assays C__26693319_10 and C__15946974_10, respectively (Thermo Fisher, Madrid, Spain).

Mutation analysis

In all patients, *JAK2V617F* was the initial mutation investigated using DNA obtained from granulocytes or from peripheral whole blood, as previously described [30, 31]. Quantification of the *JAK2V617F* allele burden was assessed in DNA by real-time allele-specific polymerase chain reaction with probes specific for the mutated and the wild-type (WT) forms, as we described [32]. The *JAK2V617F* homozygous and heterozygous states were defined as a mutant allele burden of >50% or ≤50%, respectively. In *JAK2*-WT ET and MF patients, *CALR* exon 9 and *MPL* mutations (p.W515L/K/R/A and p.S505N) were tested by PCR fragment analysis by capillary electrophoresis [33, 34]. *JAK2* exon 12 mutations were assessed by Sanger sequencing in patients with suspicion of PV and the absence of *JAK2V617F*.

Next-generation sequencing (NGS)-based gene panel test (Qiagen, Hilden, Germany) was performed on DNA extracted from mononuclear cells or whole blood, as reported previously [32] (see Supplementary online material for details regarding samples, patients, and methods). From the 26 genes tested in the panel, only the presence of mutations in *ASXL1*, *CBL*, *EZH2*, *IDH2*, *SF3B1*, *SH2B3*, *SRSF2*, *TP53*, *U2AF1*, or *ZRSR2* were considered as high molecular risk (HMR) mutations [4, 5].

Animal model

Experiments with animals conformed to EU Directive 2010/63EU and Recommendation 2007/526/EC, enforced in Spanish law under Real Decreto 53/2013. *MiR-146a*^{-/-} mice (Jackson laboratory, Bar Harbor, ME) were backcrossed in a C57BL/6J CD45.2 background for more than eight generations. Inbred WT littermates were used as controls. *MiR-146a*^{-/-} mice were monitored for signs of illness, and those showing signs of being moribund, having more than 10% weight loss or having massive palpable splenomegaly were sacrificed. The study was approved by the local ethical committee (ID# CEEA 330/2017).

Histopathology

Femur and spleen samples were fixed in 4% paraformaldehyde overnight, embedded in paraffin, and stained with hematoxylin and eosin, periodic acid–Schiff or silver impregnation to assess fibrosis. Pictures were taken at ×2.5, ×10, and ×40 magnification on a Leica 6000B inverted microscope (Leica, Madrid, Spain) equipped with a Leica DCF 320 color digital camera.

Western blotting

Protein lysates were obtained from the granulocytes of 2- and 9-month-old *miR-146a*^{-/-} mice and 9-month-old WT mice. Normalization was based on the amount of protein loaded. Total protein (12 µg) was separated by SDS–PAGE (8%) under reducing conditions. Gels were transferred onto PVDF membranes (GE Healthcare, Barcelona, Spain). Blots were blocked for 1 h in 5% PBS–BSA and incubated overnight at 4 °C with primary antibodies: anti-Sta3 (1:1000), anti-p-Stat3 (Tyr705) (1:1000) (Cell Signaling Technology, Leiden, The Netherlands), and anti-α-tubulin (1:5000) (Sigma-Aldrich, Madrid, Spain). After washing, membranes were incubated with appropriate secondary antibodies labeled with peroxidase (1:10000, GE Healthcare, Madrid, Spain). Proteins were detected using the ECL Prime Detection kit and ImageQuant LAS 4000 Imager (GE Healthcare, Barcelona, Spain). Densitometric analyses were performed using ImageJ software (<https://imagej.nih.gov/ij/>).

Statistical analyses

Estimation of the power of the study to detect an association between SNPs and MPN was evaluated using the genetic power calculator available at <http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize>. Haplotype frequencies were estimated from genotype frequencies using the expectation–maximization algorithm encoded into the SNPStats program [35]. All statistical analyses, including the Hardy–Weinberg equilibrium test, linkage disequilibrium estimation, and association of SNP genotypes and haplotypes with MPN, were performed using SNPStats software [35]. SNPStats is a free web-based tool (<https://www.snpsstats.net/start.htm>) to perform analysis of association studies based on SNPs or biallelic markers. Both the selection of analysis and the output were designed from a genetic epidemiology perspective. ORs and 95% confidence intervals (CIs) were calculated for each group compared with the homozygous genotype for the risk allele; *p* values were derived from the χ^2 test.

Continuous variables are presented as the mean ± SD or median (interquartile range, IQR), as appropriate, and categorical variables are presented as percentages. Comparisons of categorical variables between groups were carried out using the χ^2 test for tables, while numerical variables were compared using the two-tailed Student's *t* test or Mann–Whitney *U* test, where appropriate. Shapiro–Wilk test and Levene's test were used to check normal distribution and homoscedasticity assumptions, respectively. Time to event (MF transformation) was calculated from the date of PV or ET diagnosis to the date of progression to overt MF.

However, since the *JAK2*V617F allele burden was measured at different times from diagnosis and assuming a potential effect of time on the *JAK2*V617F allele burden [1, 2, 36, 37], MF-free survival according to the *JAK2*V617F allele burden was also carried out from the date of *JAK2* assessment, as previously described [2, 37]. Among PV and ET patients, secondary MF-free survival analysis was assessed by the Kaplan–Meier method, followed by the log-rank test and the Breslow–Wilcoxon method, which gives more weight to events at early time points, and, therefore, it is more likely to detect early differences between survival curves [38]. The independent effect of each variable on prognosis was calculated using a Cox proportional hazards regression model, and only the values that showed *p* values < 0.05 in the univariate analysis were incorporated into the multivariate model. Finally, to study the interaction between rs2431697 and *JAK2*V617F, this last variable was dichotomized into high and low allele burden (>50% vs. (≤50% WT)], and the interaction between TT genotype and *JAK2* (product of both variables) was evaluated by Cox regression.

Results

Association of the miR-146a rs2431697 SNP with MF

We studied 967 MPN patients (50% male; median age, 66 years, IQR 53–75) whose biological characteristics are summarized in Supplementary Table 1. The distributions of the genotype frequencies for rs2431697 and rs2910164 in the overall MPN and control populations are reported in Table 1. Both miR-SNP genetic frequencies were in Hardy–Weinberg equilibrium (not shown). No significant differences in genotype distribution were noted between patients and controls for rs2910164. In contrast, the rs2431697 TT genotype was significantly more frequent in MF patients (*n* = 314) than in controls (*n* = 600) (OR = 1.36; CI: 1.01–1.83, *p* = 0.04) (Table 1). These differences were more pronounced in the subgroup of patients with secondary MF (*n* = 134) (OR = 1.49, CI: 1.0–2.21; *p* = 0.05) (Table 2). In fact, taking as reference the population at

risk of transformation [PV (*n* = 320) and ET (*n* = 333)], we observed an enrichment of subjects carrying the TT genotype in the secondary MF group (OR = 1.50; CI: 1.01–2.23, *p* = 0.04) (Table 2). Altogether, our findings suggest that the rs2431697 TT genotype is associated with secondary MF. Since this association, according to that described in [24], might be due to lower miR-146a expression levels among TT carriers [24], we evaluated miR-146a expression in whole blood samples of rs2431697 homozygous PV (*n* = 28) and ET (*n* = 37) patients at the time of diagnosis. As expected, TT patients (*n* = 34) showed lower miR-146a levels than CC carriers (*n* = 31), with borderline significance (*p* = 0.05) (Supplementary Fig. 1).

rs 2431697 SNP as a biomarker of early progression to secondary MF

Once we confirmed that rs2431697 played a role in susceptibility to the development of secondary MF, our next step was to assess the potential relationship between this miR-SNP and the time to disease progression. From the cohort of PV and ET patients with available rs2431697 genotype data (*n* = 653), we had complete clinical information at baseline and during the follow-up of those enrolled from two of the participating hospitals [Hospital Morales Meseguer (Murcia) and Hospital del Mar (Barcelona)]. After reviewing the clinical information and the BM histology of all patients from these two hospitals, only those with a confirmed diagnosis of PV (*n* = 112) and ET (*n* = 120) according to the current revised 2016 WHO criteria [39] were found to be eligible for this analysis.

Table 3 summarizes the clinical and biological baseline characteristics of these 232 patients. With a median follow-up time of 8.5 years (range, 0.1–24.4 years), 18 patients (7.8%) progressed to MF. Most of the MF transformations were observed in PV patients: 14 out of 112 (12.5%) PV patients compared with 4 out of 120 (3.3%) ET patients (*p* = 0.01). Indeed, the median time to transformation was shorter in PV patients (19.1 years, 95% CI: 15.6–22.5 years) than in ET patients (not reached, 95% CI: not reached) (*p* = 0.004) (Fig. 1a). As expected, the risk of secondary MF increased with the duration of the disease. Thus, the

Table 1 miR-146a SNP genotype frequencies (rs2431697 and rs2910164) in overall MPN population and controls.

	Controls	PV	ET	MF	PMF	Post-PV/ET MF
rs2431697	<i>n</i> = 600 (%)	<i>n</i> = 320 (%)	<i>n</i> = 333 (%)	<i>n</i> = 314 (%)	<i>n</i> = 180 (%)	<i>n</i> = 134 (%)
CC	127 (21)	70 (22)	82 (25)	68 (22)	42 (23)	26 (19)
TC	313 (52)	168 (52)	160 (48)	142 (45)	81(45)	61(46)
TT	160 (27)	82 (26)	91 (27)	104 (33)	57 (32)	47 (35)
rs2910164	<i>n</i> = 600 (%)	<i>n</i> = 319 (%)	<i>n</i> = 318 (%)	<i>n</i> = 310 (%)	<i>n</i> = 179 (%)	<i>n</i> = 131 (%)
CC	38 (6)	24 (7)	22 (7)	25 (8)	16 (9)	9 (7)
GC	208 (35)	124 (39)	123 (39)	114 (37)	66 (37)	48 (37)
GG	354 (59)	171 (54)	173 (54)	171 (55)	97 (54)	74 (56)

Table 2 Genotype association analysis of miR-146a rs2431697 in MPN patients.

TT vs. CC + CT	PV <i>n</i> = 320 vs. controls <i>n</i> = 600	ET <i>n</i> = 333	MF <i>n</i> = 314	PMF <i>n</i> = 180	Post-PV/ET MF <i>n</i> = 134	Post-PV/ET MF <i>n</i> = 134 vs. PV + ET <i>n</i> = 653
OR (95% CI)	0.95 (0.70–1.29)	0.97 (0.73–1.31)	1.36 (1.01–1.83)	1.27 (0.89–1.83)	1.49 (1.00–2.21)	1.50 (1.01–2.23)
<i>p</i> value	0.73	0.83	<0.05	0.19	0.05	<0.05

Significant values are shown in bold.

Table 3 Main clinico-hematological characteristics of PV and ET patients at the time of diagnosis.

	PV <i>n</i> = 112	ET <i>n</i> = 120	All <i>n</i> = 232
Age, years ^a	64 (52–74)	57 (39–73)	62 (46–74)
Female sex, <i>n</i> (%)	52 (46)	78 (65)	130 (56)
Male sex, <i>n</i> (%)	60 (54)	42 (35)	102 (44)
Hemoglobin, g/l	17.5 ± 1.7	14.1 ± 1.5	15.7 ± 2.4
WBC count, ×10 ⁹ /l	11.5 ± 4.4	8.7 ± 3.2	10.0 ± 4.1
Platelet count, ×10 ⁹ /l	597 ± 288	816 ± 289	712 ± 308
<i>JAK2</i> mutated, <i>n</i> (%)	112 (100)	70 (58.3)	182 (78.4)
V617F Heterozygous	84 (75)	70 (58.3)	154 (66.4)
V617F Homozygous	25 (22.3)		25 (10.8)
Exon 12	3 (2.7)	–	3 (1.3)
<i>JAK2</i> wild-type, <i>n</i> (%)	0 (0)	50 (41.7)	50 (21.6)
<i>CALR</i> mutated, <i>n</i> (%)		25 (20.8)	25 (10.8)
Type 1		10 (8.3)	
Type 2		9 (7.5)	
Others		6 (5.0)	
<i>MPL</i> mutated, <i>n</i> (%)		2 (1.6)	2 (0.9)
Triple negative, <i>n</i> (%)		23 (19.2)	23 (9.9)
Bone marrow fibrosis ^b			
MF-0, <i>n</i> (%)	51 (67.1)	87 (72.5)	138 (70.4)
MF-1, <i>n</i> (%)	25 (32.9)	33 (27.5)	58 (29.6)
Follow-up time median, yrs (range)	9 (0.1–24)	8 (0.1–22)	8.5 (0.1–24)

^aMedian (IQR: interquartile range); cellular counts (hemoglobin, WBC, and platelets) are expressed as median ± standard deviation (SD).^bOut of 196 assessable patients.

cumulative incidence of secondary MF in our series was 4%, 11%, and 19% at 5, 10, and 15 years, respectively, in PV patients and 0%, 1%, and 7% at the same time intervals in ET patients (Supplementary Table 2), consistent with previous reports [1, 40].

To further confirm the representative nature of our cohort, we evaluated the contributions of the driver mutations and *JAK2*V617F allele burden in MF progression. All patients who progressed to post-PV MF (*n* = 14) carried the *JAK2*V617F mutation, whereas among those who progressed to post-ET MF (*n* = 4), 1 (25%) and 3 (75%)

carried the *JAK2*V617F and *CALR* mutations, respectively. Of note, the median time between diagnosis and sample acquisition was 3 months. In addition, 46% of patients were sampled within 1 month of diagnosis, and 62% were sampled within the 1 year of diagnosis. As expected, the estimated 10-year MF-free survival was significantly shorter for *JAK2*V617F homozygous patients (62%; 95% CI: 61.7–62.3) than for *JAK2*V617F heterozygous patients (90.9%; 95% CI: 90.8–90.95) or those with wild-type *JAK2* (94.5%; 95% CI: 94.4–94.6) (*p* = 0.01, Fig. 1b). Similar results were observed when we evaluated MF-free survival according to the *JAK2*V617F status from diagnosis (*p* = 0.01, Supplementary Fig. 2).

We next evaluated the effect of rs2431697 on MF progression. First, we studied the genotype distribution in our cohort of 232 patients with chronic-phase MPN (PV and true-ET) (Table 4). Of note, both groups (TT, *n* = 66, CC + CT, *n* = 166) were similar in terms of age and sex distributions, phenotype (PV/ET), driver mutations (*JAK2*V617F, *CALR*, and *MPL*), and *JAK2*V617F allele burden. No significant differences were observed in the leukocyte count, the hemoglobin level, or BM fibrosis. Patients with rs2431697 TT presented a median MF-free survival significantly shorter than the other group (CC + CT) (19 years [95% CI: 10–28] vs. median time not reached, *p* = 0.016) (Fig. 1c). The major differences were observed at early time points (Breslow–Wilcoxon test, *p* = 0.001). In fact, 4 out of 10 TT patients (40%) who transformed to secondary MF progressed in the first 5 years from diagnosis compared with 0 out of 8 in the CC + CT group (Fig. 1c). In the first 10 years from diagnosis, most rs2431697 TT patients (7/10, 70%) progressed to secondary MF vs. 25% (2/8) among CC + CT patients. Thus, the estimated 10-year MF-free survival was longer for CC + CT patients (97.4%, 95% CI 97.36–97.43) than for TT patients (84.9%, CI 84.79–85.01) (HR 3.00, 95% CI 1.18–7.64; *p* = 0.02).

Independent role of rs2431697 in the transformation to MF

To confirm the independent role of rs2431697 in the transformation to MF, we performed a multivariate analysis

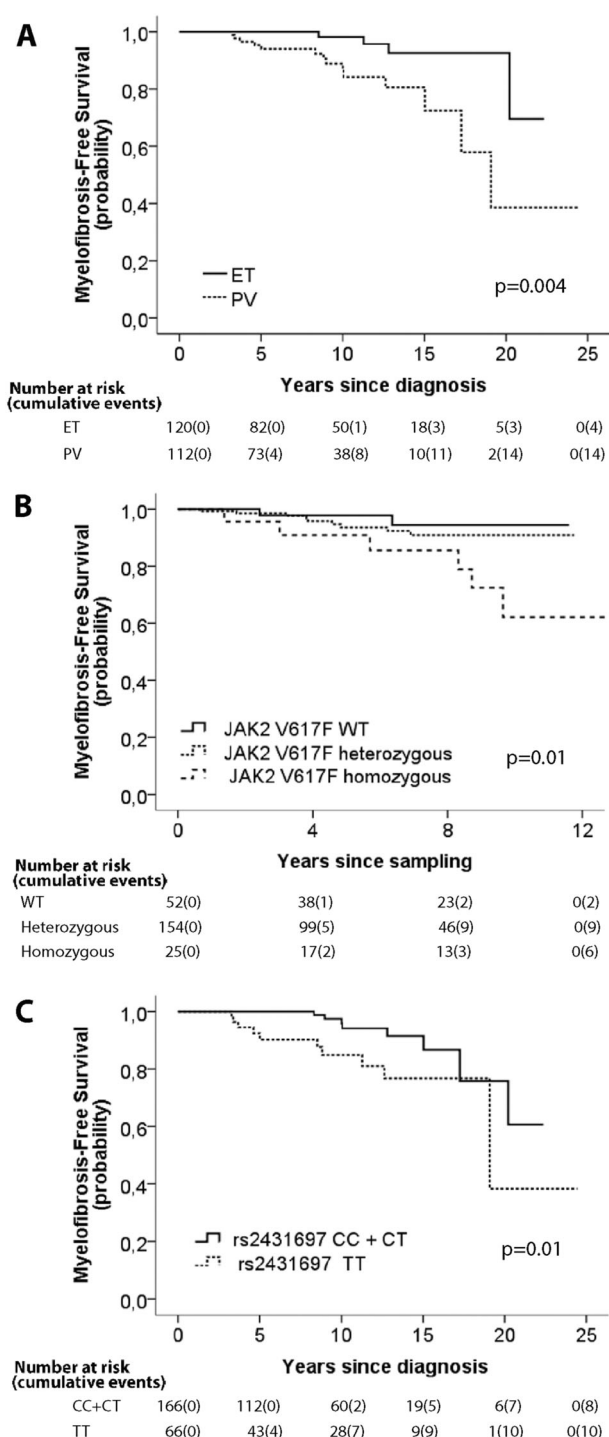


Fig. 1 Myelofibrosis-free survival probability. **a** Kaplan–Meier estimate of the MF-free survival of 232 patients with a diagnosis of PV (dotted line, $n = 112$) or true-ET (solid line, $n = 120$) ($p = 0.004$). **b** Kaplan–Meier estimate of the MF-free survival of chronic-phase cMPN patients (PV or True-ET, $n = 232$) according to the *JAK2*V617F mutational status: wild type (solid line), heterozygous (dotted line), and homozygous (striped line). Observations were left-censored at the time of *JAK2* assessment ($p = 0.01$). **c** MF-free survival of patients with MPNs in the chronic phase (PV and true-ET) according to the rs2431697 genotype. Solid and dotted lines represent CC + CT ($n = 166$) and TT ($n = 66$) patients for rs2431697, respectively.

Table 4 Clinical and hematological features of 232 patients with PV and true-ET stratified by the rs2431697 genotype.

		CC + CT <i>n</i> = 166	TT <i>n</i> = 66	<i>p</i> value
ET, <i>n</i> (%)	120	87 (52.4)	33 (50)	
PV, <i>n</i> (%)	112	79 (47.6)	33 (50)	0.77
Age, median (IQR)	232	59 (48–74)	63 (41–74)	0.42
>60 years old, <i>n</i> (%)		87 (52.4)	35 (53)	1
Female sex, <i>n</i> (%)	130/232	90 (54.2)	40 (60.6)	0.47
Hemoglobin (median $\pm$ SD, g/l)	232	15.6 $\pm$ 2.2	15.9 $\pm$ 2.6	0.34
<12 g/l, <i>n</i> (%)		5 (3.01)	2 (3.03)	1
WBC (median $\pm$ SD)	232	10.1 $\pm$ 4.5	9.6 $\pm$ 2.6	0.27
>11 $\times 10^9/l$, <i>n</i> (%)		50 (31.1)	17 (27.9)	0.74
Platelets $\times 10^9/l$ (median $\pm$ SD)	232	737 $\pm$ 317	645 $\pm$ 273	0.045
<i>JAK2</i>	232			
Wild type, <i>n</i> (%)		37 (22.3)	13 (19.7)	0.72
Mutated ^a , <i>n</i> (%)		129 (77.7)	53 (80.3)	
<i>JAK2</i> V617F hetero, <i>n</i> (%)		110 (87.3)	44 (83.0)	0.48
<i>JAK2</i> V617F homo, <i>n</i> (%)		16 (12.7)	9 (17.0)	
CALR mutated, <i>n</i> ^b (%)	50	16/37 (43.2)	9/13 (69.2)	0.2
MPL mutated, <i>n</i> ^b (%)	25	1/21 (4.8)	1/4 (25.0)	0.3
Triple negative, <i>n</i> ^b (%)	25	20/21 (95.2)	3/4 (75.0)	0.3
Grade 1 BM fibrosis, <i>n</i> (%)	196	35/138 (25.4)	23/58 (39.7)	0.06
Additional genetic mutations				
Available	100/232	70 (42.2)	30 (45.4)	
Detected	49/100	34 (48.6)	15 (50.0)	1
No. of mutated genes				
0	50	36 (51.4)	14 (46.7)	0.85
1	36	25 (35.7)	11 (36.7)	
≥ 2	14	9 (12.9)	5 (16.7)	
No. of mutations				
0	51	36 (51.4)	14 (46.7)	0.54
1	33	24 (34.3)	9 (30)	
≥ 2	17	10 (14.3)	7 (23.3)	
Patients with HMR mutations	23/100	16 (22.9) ^c	7 (23.3) ^d	1

IQR interquartile range, HMR high molecular risk.

^a3 exon 12 positive.

^b*n* out of assessable patients.

^c*ASXL1* ($n = 7$); *SH2B3* ($n = 3$); *EZH2* ($n = 1$); *IDH2* ($n = 1$); *SF3B1* ($n = 1$); *CBL* ($n = 1$); *ASXL1* + *CBL* ($n = 1$); *ASXL1* + *CBL* + *ZRSR2* ($n = 1$).

^d*ASXL1* ($n = 2$); *SF3B1* ($n = 2$); *IDH2* ($n = 1$); *SH2B3* ($n = 1$); *TP53* ($n = 1$).

that was adjusted for a number of covariates with relevance in terms of progression to MF. Multivariate analysis confirmed that rs2431697 TT retained its prognostic value for the development of secondary MF (HR 2.94, 95% CI: 1.15–7.50, $p = 0.02$) independent of the initial diagnosis (PV or ET), *JAK2* mutational status (mutated vs. non-mutated), or *JAK2*V617F allele burden (Table 5).

To further explore the rs2431697 independent effect on MF-free survival, we performed a Kaplan–Meier analysis,

Table 5 Univariate and multivariate analysis of risk factors for secondary MF in 232 patients with PV or true-ET.

	Univariate		Multivariate	
	HR (95% CI)	p value	HR (95% CI)	p value
Age >60 year	0.84 (0.32–2.18)	0.72	–	
Male sex	0.83 (0.32–2.17)	0.71	–	
PV (vs. ET)	4.5 (1.48–13.74)	0.008	4.43 (1.45–13.5)	0.009
Hemoglobin <12 g/dl	1.31 (0.16–10.37)	0.80	–	
WBC count >11 × 10 ⁹ /l	0.95 (0.33–2.77)	0.93	–	
Grade 1 bone marrow fibrosis	1.72 (0.63–4.69)	0.29	–	
JAK2 wild-type (vs. mutated)	0.50 (0.14–1.72)	0.27	–	
JAK2V617F allele burden ^a	2.61 (1.19–5.72)	0.016	1.62 (0.61–4.33)	0.33
Rs2431697 TT vs. CT + CC	3.00 (1.18–7.64)	0.021	2.94 (1.15–7.50)	0.02

^aWild-type-hetero-homozygous.
Significant values are shown in bold.

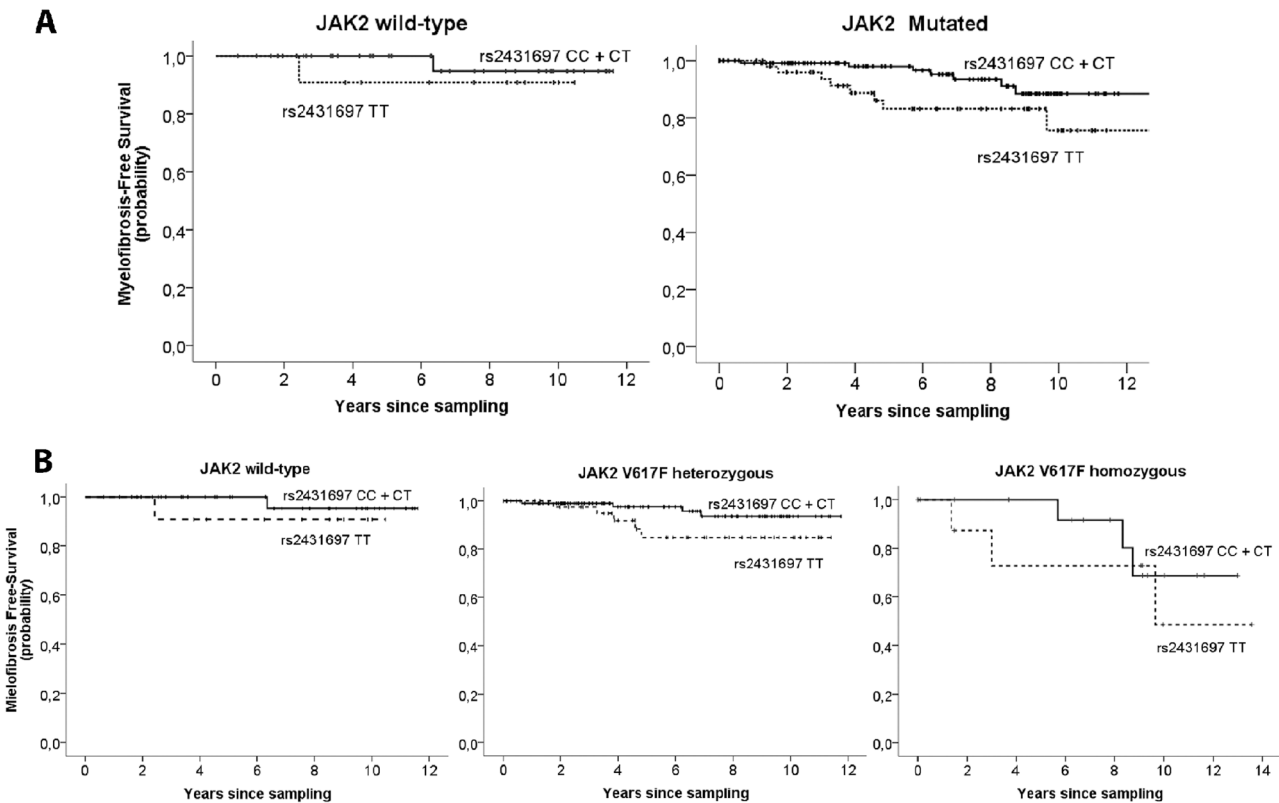


Fig. 2 Myelofibrosis-free survival probability according to the *MIR-146A* SNP rs2431697 genotype. Estimation was stratified by (a) the *JAK2* mutational status (wild-type vs. mutated, $p = 0.03$; Breslow–Wilcoxon test, $p = 0.01$) or (b) the *JAK2V617F* allele burden (wild-type; *JAK2V617F* heterozygous; *JAK2V617F* homozygous, $p =$

0.05; Breslow–Wilcoxon test, $p = 0.04$). As the *JAK2V617F* allele burden was measured at different times since diagnosis, observations were left-censored at the time of the *JAK2* assessment. Solid and dotted lines represent rs2431697 CC + CT ($n = 166$) and TT ($n = 66$) patients, respectively.

according to the rs2431697 genotype (TT vs. CC + CT) stratified by the *JAK2* mutational status (wild-type vs. mutated) or by the *JAK2V617F* allele burden [wild type, low allele burden (<50%) or high allele burden ($\geq 50\%$)]. We observed an adverse prognostic effect of the rs2431697 TT genotype in both *JAK2*-mutated and *JAK2* wild-type patients (Fig. 2a). Thus, the cumulative incidence of

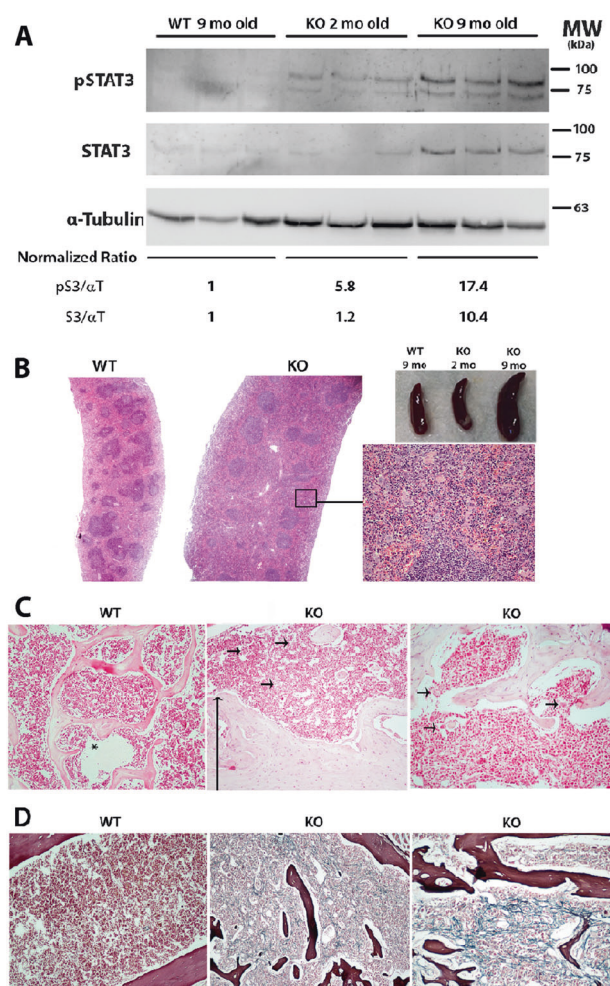
secondary MF at 10 years was higher in rs2431697 TT than in CC + CT genotype carriers in both patients with mutated *JAK2V617F* (24.5% vs. 11.5%) and those with wild-type *JAK2* (9.1% vs. 5.3%; $p = 0.03$ and $p = 0.01$ for log-rank and Breslow–Wilcoxon test, respectively). Among all of the *JAK2* mutational statuses (wild-type, low allele burden [<50%], or high allele burden [$\geq 50\%$]), the time to MF

progression was shorter for the rs2431697 TT carriers. Thus, the estimated 10-year MF-free survival in CC + CT vs. TT patients was 95.5% (95% CI 95.4–95.6) vs. 90.9% (95% CI 90.7–91.1); 93.6% (95% CI 93.5–93.7) vs. 84.8% (95% CI 84.7–84.9), and 68.8% (95% CI 68.6–69.1) vs. 48.6% (95% CI 48.1–49.9) for wild-type, heterozygous and homozygous patients, respectively ($p = 0.05$ and $p = 0.04$ for log-rank and Breslow–Wilcoxon test, respectively) (Fig. 2b). Among all patients, those who were *JAK2V617F* homozygote and rs2431697 TT showed a median time to fibrotic progression that was significantly shorter (9.6 years, 95% CI not reached) than that of any other group (median time not reached) (Fig. 2b). We also explored the interaction between the *JAK2* mutational status and the TT genotype without finding differences ($p = 0.69$), suggesting that the rs2431697 genotype does not modify the effect of *JAK2V617F* allele burden.

Our next step was to assess whether additional genetic mutations, other than *JAK2*, *CALR*, or *MPL*, were influencing our findings. With this aim, we evaluated the contribution of 26 myeloid cancer genes by NGS in 100 out of 232 patients (all those with DNA available within 36 months of diagnosis and clinical follow-up of more than 4 years) (Table 4). Importantly, the median age for patients carrying TT genotype (63.5; IQR: 46–71) was similar to CC + CT patients (63.0; IQR: 48–76) ($p = 0.5$). As expected, the most frequent variants/mutations were found in *TET2* and *DNMT3A*, independently of rs2431697 genotype (not shown). To note, TT and CC + CT patients had similar number of mutated genes and similar number of mutations (Table 4). Patients with additional mutated genes ≥ 2 had a trend toward shorter MF-free survival [HR 2.2; 95% CI: 0.91–5.41; $p = 0.08$]. Among them ($n = 14$), 0 out of 9 CC + CT and 3 out of 5 TT progressed to MF. Regarding the impact of HMR mutations, we found 26 variants in 23 patients, *ASXL1* being the most frequently mutated gene ($n = 11$) (Table 4). Importantly, the number of patients with HMR mutations was similar between TT and CC + CT patients. Finally, at the time of transformation, the frequency of additional mutations in nondriver genes was similar ($p = 0.87$) in TT and CC + CT patients (Supplementary Table 3). Overall, our findings suggest that the rs2431697 TT genotype could represent an independent prognostic marker of early progression to secondary MF.

miR-146a low levels are associated with increased inflammatory signaling in myeloproliferative neoplasm

Since miR-146a acts as a negative regulator of inflammation [21], we next examined whether MPN patients with the TT genotype have increased inflammatory cytokines. To test this, we evaluated the plasma levels of 7 cytokines related to



NF- κ B pathway in 80 MPN patients at diagnosis ($n = 59$, 74%) or before any therapy ($n = 21$; 26%) (Supplementary materials and Supplementary Table 4). Patients with TT genotype showed, compared with those with CC + CT genotype, significantly higher levels of IL-1 β and IL-1RA; and a trend toward higher levels of IL-8 ($p = 0.09$) and IL-6 ($p = 0.17$) (Supplementary Table 5).

In addition, we explored the effect of miR-146a down-regulation in SET-2 cells, a *JAK2V617F* clonal hematopoiesis in vitro model (see Supplementary materials for details). Transfection of SET-2 cells with anti-miR-146a provoked a significant increase of *IL1B* levels in comparison with cells transfected with a scrambled control (Supplementary Fig. 3), supporting our findings in patients.

miR-146a deficiency in mice results in an age-related increase in Stat3 signaling

Finally, we evaluated the potential interplay between the Tlr4/Nf- κ B pathway, regulated by miR-146a [16], and Jak/Stat signaling by measuring total Stat3 and pStat3 levels

◀ **Fig. 3** *MIR-146A* deficiency results in aging progressive Stat3 activation. **a** Immunoblot analysis of the Stat3 and p-Stat3 levels in granulocytes from 2- and 9-month-old *miR-146a*^{-/-} and 9-month-old WT mice. Cells from three different WT and KO animals were used. Proteins were normalized to α -tubulin as the internal control and quantified by densitometric analysis using the ImageJ program (mo = months). **b** Splenomegaly and extramedullary hematopoiesis in *miR-146a*^{-/-} mice. Upper right: gross anatomy of spleens from a single representative 9-month-old wild-type (WT) or 2- and 9-month-old *miR-146a*^{-/-} mice. Left: representative H&E-stained spleen sections from WT and *miR-146a*^{-/-} (KO) mice (magnification $\times 2.5$). Note the expansion of red pulp and the lack of definition in the separation between white and red pulp. Lower right: detail at higher magnification ($\times 40$). Note the infiltration by megakaryocytes, erythroblasts, and erythroid islands as a sign of extramedullary hematopoiesis. **c** Myeloproliferation, fibrosis, and new bone formation in *miR-146a*^{-/-} deficient bone marrow. Left: representative H&E-stained femur sections from WT 9-month-old mice ($\times 10$ magnification). Note the adipose tissue (*) in the normal hematopoietic tissue. Megakaryocytes are numerous and clearly visible. Center: representative H&E-stained femur sections from *miR-146a*^{-/-} 9-month-old mice ($\times 10$ magnification). The bone marrow is markedly hypercellular. Megakaryocytes are increased and are small and dysplastic (arrows). Note new bone formation (indicated by the length of the vertical arrow). Right: detail at higher magnification ($\times 40$). Note the expansion of the myeloid line and megakaryocytic dysplasia (arrows). As a result of the underlying fibrosis, the hematopoietic cells appear to be lined up. **d** Gomori's silver reticulin staining of representative longitudinal sections of the femur from WT and *miR-146a*^{-/-} 9-month-old mice. Note the increased reticulin (black stain) at $\times 10$ (center) and $\times 40$ (right) magnification in *miR-146a*^{-/-} mice compared with WT (left, $\times 10$ magnification). Similar results were obtained from at least three animals per group.

in granulocytes from WT ($n = 3$) or *miR-146a*^{-/-} mice ($n = 6$). As shown in Fig. 3, we observed higher levels of total Stat3 and pStat3 in *miR-146a*^{-/-} mice than in WT mice. Differences were found beginning in the second month of life and increased with aging. Importantly, at 9 months of age, the increase in Stat3 activation was associated with anemia, myeloid proliferation, BM fibrosis, progressive splenomegaly, and extramedullary hematopoiesis (Fig. 3 and Supplementary Fig. 4).

Discussion

The present study provides the first evidence that miR-146a, a critical molecular brake on inflammation, plays a role in the progression of MPN from the chronic phase to MF. Solid evidence suggests that dysregulated expression of miR-146a participates in malignant transformation and cancer progression [41] by promoting NF- κ B signaling by targeting *TRAF6* and *IRAK1* [21, 42]. In hematological disorders, miR-146a plays an important role in myelopoiesis [42]. In mice, miR-146a deficiency drives a chronic inflammatory phenotype with progressive myeloproliferation involving the spleen and BM and resembling human MPN in the end-stage marrow fibrosis [16, 18]. Indeed,

injections of conjugated miR-146a mimic to *miR-146a*^{-/-} mice have been reported to prevent NF- κ B activation in myeloid cells and to mitigate myeloproliferation [43]. In addition, simultaneous knockdown of miR-145 and miR-146a in mouse hematopoietic stem cells induces thrombocytosis and dysplastic megakaryopoiesis [23], common features between 5q-syndrome and MF. In humans, other studies confirmed decreased miR-146a levels in the CD34+ cells of myelodysplastic syndrome patients with isolated del (5q) compared with the levels detected in normal CD34+ cells [44]. However, to the best of our knowledge, the role of miR-146a in MPN has not been previously demonstrated.

Among the SNPs that regulate miR-146a expression levels [24], we studied the frequencies of rs2431697 and rs2910164 in controls and MPN patients. Compared with the control subjects, MF patients, particularly secondary MF patients, showed the highest frequency of the rs2431697 TT genotype. In addition, the rs2431697 TT genotype was significantly more frequent in post-PV/ET MF patients than in the at-risk population (PV and ET patients). This observation underlines the role of inflammation as a driver of clonal expansion from the early disease stage to the myelofibrotic burn-out phase [6]. In line with our results, Masselli et al. have recently identified the 2518 A/G polymorphism in MCP-1, the main chemotactic factor for monocyte migration to sites of inflammation, as a potential genetic predisposition factor for secondary MF [45].

Importantly, our findings suggest that the rs2431697 TT genotype not only increases susceptibility to secondary MF, but may also be considered a marker for early progression. Although *JAK2V617F* homozygosity or a high *JAK2V617F* allele burden are unequivocal risk factors for secondary post-PV MF [1, 5] and although type 1-*CALR* mutations are unequivocal risk factors for post-ET MF [46], the *JAK2V617F* allele burden changes throughout the natural history of the disease and as a consequence of the treatments [32]. Moreover, fibrotic progression in patients with *CALR* mutations is a late event during the course of the disease [46]. Thus, the discovery of new molecular markers of fibrotic progression at the time of PV/ET presentation could allow better risk stratification and be used to assess the adequacy of earlier therapeutic interventions. In this framework, we observed a strong effect of the miR-146a rs2431697 TT genotype on the risk of MF transformation after correction for original diagnosis (PV/ET) and the *JAK2* allele burden in a contemporary series that included 232 patients diagnosed according to the WHO 2016 criteria. Our data suggest that the rs2431697 TT genotype could identify a subgroup of patients with a higher risk of progression to MF independent of the *JAK2V617F* allele burden. In this sense, miRNA therapeutics in selected patients with a higher risk of MF transformation are imaginable in the future.

In line with our results, rs2431697 has also been associated with other inflammatory conditions [27] and with systemic lupus erythematosus susceptibility [24]. Mechanistically, Lofgren et al. showed that the TT genotype of this miR-SNP, located in a putative regulatory region for *MIR146A*, leads to lower gene transcription [24]. Consistent with that study, we observed that among MPN patients in chronic phase (PV and ET), TT carriers displayed a tendency toward lower miR-146a expression levels (Supplementary Fig. 1). In a previous study, we demonstrated that, in normal hematopoiesis, under inflammatory stress, monocytes from rs2431697 TT individuals had an increased pro-inflammatory response with higher *IL6* expression levels than CC subjects [27]. In the current study, our in vitro and in vivo studies showed that, in the context of a clonal *JAK2* hematopoiesis, the downregulation of miR-146a is associated with an increase of inflammatory cytokines. Interestingly, IL-1 β and IL-1RA have been found increased in PMF compared with PV [47]. Furthermore, in PV, increased levels of IL-1 β correlated with fibrotic transformation [48]. Panteli et al. showed increased IL-6 levels in all patients who transformed to secondary MF [49]. Since MPN are inflammatory diseases [6], we speculate that the lower levels of miR-146a observed in rs2431697 TT individuals may contribute to an increased inflammatory microenvironment that promotes progression to overt MF from chronic-phase disease (PV or ET). Supporting this hypothesis, our results show, for the first time, that miR-146a, a negative modulator of the Tlr/NF- κ B axis [16], regulates both Stat3 expression and activation in hematopoietic cells. Overall, these data indicate that in a mouse model of inflammation without driver MPN mutations (*miR-146a*^{-/-} mice), the aging MF-like phenotype (myeloproliferation, splenomegaly, progressive BM fibrosis, and extramedullary hematopoiesis) is associated with progressive Jak/Stat3 activation in the myeloid line.

Additional evidence supports cross-communication between the JAK/STAT3 and NF- κ B/miR-146a pathways [50, 51]. Notably, Stickel et al. have recently demonstrated that after allogeneic hematopoietic cell transplantation, the lack of miR-146a in host dendritic cells exacerbates acute graft-vs.-host disease via JAK/STAT signaling [52]. Similarly, in diffuse B-cell lymphoma, the overproduction of IL-6 and/or IL-10 due to aberrant activity of NF- κ B triggers the JAK/STAT3 pathway in an autocrine way [53]. Finally, a recent study has shown in a mouse model that BM-specific loss of *ABI-1* is linked to a hyperactive STAT3/NF- κ B signaling [54] and induces an MPN-like phenotype with features resembling human MF [54].

There are some limitations regarding this study that we must acknowledge. Especially, our sample size was not large enough to allow a split into learning and validation cohorts. Therefore, further studies are needed to confirm our results.

In summary, our findings are the first to demonstrate a role for miR-146a in the pathogenesis and progression of MPN. In particular, we identified the rs2431697 TT genotype as a potential genetic biomarker of fibrotic progression from chronic-phase MPN. In addition, our data link the lower levels of miR-146a to an increase in Stat3 signaling in the myeloid population. These findings strongly suggest that the rs2431697 genotype represents a new prognostic factor for MPN, and they point to the utility of the development of new therapeutic strategies for these patients based on the use of novel agents such as miRNA mimics.

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Author contributions ABA, BB, LZ, RTM, EJC, RC, AMR-G, and FFM collected the data and performed the molecular studies. BB and CFR performed the NGS studies. ABA, EJC, AMR-G, and FFM performed ELISAs and Luminex assay. IA performed histological studies. ABA, BB, LZ, AAL, JCHB, JMHR, RGC, and CM analyzed and interpreted the results and wrote the paper. ABA, BB, LZ, JCHB, JMHR, EL, CGH, AK, DVF-S, MTG-C, RA, CB, PV, VGG, BA, NE, RC, EJC, and RTM collected the data and approved the final version. FFM, RGC, and CM designed the study. ABA, EJC, RTM, and FFM performed the statistical analysis. All authors participated in drafting the paper and approved the final version of the paper for submission.

Compliance with ethical standards

Conflict of interest This study was partially funded by Novartis International (CINC424AES05T).

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