original research report

The mutation profile of *JAK2*, *MPL* and *CALR* in Mexican patients with Philadelphia chromosome-negative myeloproliferative neoplasms

Nancy Labastida-Mercado [a,b], Samantha Galindo-Becerra [a,b], Javier Garcés-Eisele [b,c], Perla Colunga-Pedraza [d], Valeria Guzman-Olvera [b], Virginia Reyes-Nuñez [c], Guillermo J Ruiz-Delgado [a,b,c], Guillermo J Ruiz-Argüelles [a,b,c,e,*]

[a] Centro de Hematología y Medicina Interna de Puebla, Mexico, [b] Universidad Popular Autónoma del Estado de Puebla, Mexico, [c] Laboratorios Clínicos de Puebla, Mexico, [d] Universidad Autónoma de Nuevo León, Mexico, [e] Universidad de Las Américas Puebla, Mexico

[*] Corresponding author at: Centro de Hematología y Medicina Interna de Puebla, 8B Sur 3710, 72530 Puebla, PUE, Mexico. Tel.: + 52 (222) 243 8100; fax: + 52 (222) 243 8428. · gruiz1@clinicaruiz.com · Received for publication 24 September 2014 · Accepted for publication 9 December 2014

Hematol Oncol Stem Cell Ther 2015; 8(1): 16–21

DOI: http://dx.doi.org/10.1016/j.hemonc.2014.12.002

CONTEXT AND OBJECTIVE: By using molecular markers, it is possible to gain information on both the classification and etiopathogenesis of chronic myeloproliferative neoplasias (MPN).

METHODS: In a group of 27 Mexican mestizo patients with MPNs, we studied seven molecular markers: the BCR/ABL1 fusion gene, the JAK2 V617F mutation, the JAK2 exon 12 mutations, the MPL W515L mutation, the MPL W515K mutation, and the calreticulin (CALR) exon 9 deletion or insertion. Patients with the BCR/ABL1 fusion gene were excluded. We studied 14 patients with essential thrombocythemia (ET), eight with polycythemia vera (PV), four with primary myelofibrosis (MF), and one with undifferentiated MPN.

RESULTS: We found twelve individuals with the JAK2 V617F mutation; five of them had been clinically classified as PV, five as ET, and one as MF. One patient with the MPL W515L was identified with a clinical picture of ET. Five patients with the CALR mutation were identified, four ET and one MF. No individuals with either the MPL W515K mutation or the JAK2 exon 12 mutations were identified. The most consistent relationship was that between PV and the JAK2 V617F mutation ($p = .01$).

CONCLUSIONS: Despite its small size, the study shows much less prevalence of JAK2 mutation in PV, ET and MF, which does not match international data.

KEYWORDS: Myeloproliferative neoplasms; Molecular markers; JAK2 V617F mutation; JAK2 exon 12 mutation; MPL W515L mutation; MPL W515K mutation; CAL-R mutations

INTRODUCTION

Chronic myeloproliferative neoplasias (MPN) are clonal hematopoietic stem cell disorders characterized by proliferation of myeloid cell lineages in the bone marrow and increased numbers of mature and immature cells in the blood.[1,2] MPNs include polycythemia vera (PV), essential thrombocythemia (ET), idiopathic myelofibrosis (MF) and chronic myelogenous leukemia (CML), plus rarer subtypes such as chronic neutrophilic leukemia, hypereosinophilic syndrome, and chronic eosinophilic leukemia. These diseases overlap with myelodysplastic/myeloproliferative diseases such as atypical CML and chronic myelomonocytic leukemia, in which proliferation is accompanied by dysplastic features or ineffective hematopoiesis in other lineages.[3,4] Although there are stringent diagnostic criteria for MPN subtypes, precise categorization remains a subject of debate and, furthermore, it can be difficult to differentiate some cases from reactive disorders. CML is characterized by a pathognomonic

molecular marker, the BCR-ABL1 fusion, and the primary abnormalities driving excess proliferation in most other cases have been obscure. Several lines of evidence have implicated aberrant protein kinase (PTK) signaling as the root cause of several MPNs.[3,4]

Mutations in JAK2, MPL and calreticulin (CALR) are highly relevant to the Philadelphia chromosome (Ph1)-negative myeloproliferative neoplasms. In 2005, the first recurrent molecular abnormality was described, consisting of a G > T point mutation in JAK2 (JAK2{NM_004972.2}:c.2343G > T) and resulting in a valine to phenylalanine substitution at residue 617 (V617F).[5] Subsequently, the discovery of mutations in MPL, represented by a W > L (MPL{NM_005373.2}:c.1544G > T) or W > K (MPL:c.1543_1544TG > AA) shift at codon 515, and of variable molecular abnormalities (point mutation, insertion, deletion) in JAK2 exon 12 was also reported.[6] Almost all patients with PV have a somatic genetic defect in JAK2 that is represented by the V617F allele in 90–95% of cases and by abnormalities in exon 12 in roughly 2%, while they are spared by MPL mutations. On the other hand, only 60% of patients with ET or MF harbor the JAK2 V617F mutation and 3–7% exhibit the MPL W515L/K mutation.[7] Recently, somatic mutations in the gene CALR, encoding calreticulin, have been found in most patients with ET or MF with nonmutated JAK2 and MPL. Mutant CALR is a result of frameshift mutations, caused by exon 9 deletions or insertions; type-1, 52-bp deletion (p.L367fs*46), and type-2, 5-bp TTGTC insertion (p.K385fs*47) variants constitute more than 80% of these mutations.[8]

With these molecular markers, it is possible to gain information on both the classification and etiopathogenesis of some MPNs. In a group of Mexican mestizo patients with MPNs, we studied seven molecular markers of the MPNs: the BCR/ABL1 fusion gene, the JAK2 V617F mutation, the JAK2 exon 12 mutations, the MPL W515L mutation, the MPL W515K mutation, the type-1 CALR mutation and the type-2 CALR mutation. We report here the results of these investigations.

MATERIAL AND METHODS

Patients

Patients with hematological malignancies who presented at the Centro de Hematología y Medicina Interna de Puebla after August 2005 were prospectively accrued in the study along with DNA samples from our bank. The diagnoses and classification of leukemia were done according to conventional criteria[1,9,10]; patients were studied, treated and followed up by one of the authors of this study (GJRA). Informed consent was obtained from all patients. The definition of mestizo employed in this study covers individuals born in Latin America who had both Amerindian and white ancestors.[11] Included in the study were 150 healthy blood donors as the control group.

Molecular biology studies

Analysis of the BCR/ABL1 fusion gene

The BCR-ABL1 specific transcripts were detected by RT-PCR, as previously described.[12] Briefly, total RNA, purified from 1 ml peripheral blood, was reverse transcribed by standard methods. One fifth of the cDNA was amplified for 40 cycles (pre-cycle: 1 min 95 °C; cycles: 15 s 94 °C, 20 s 60 °C, 20 s 72 °C; post-cycle: 2 min 72 °C) in 50 µl final volume (2 U Taq, 1.5 mM MgCl2, 0.4 µM each primer: TCGTGTGTGAAACTCCAGAC, CCATCCCC CATTGTGATTAT, and ACTGCCCGGTTGTC GTGT). Ten microliters of amplified material were analyzed on 4.5% PAGE. Negative PCR results were validated by detection of ABL1 transcripts (primers: CCATCCCCCATTGTGATTAT and TAGCA TCTGACTTTGAGCCT).

Analysis of the JAK2 V617F mutation

An amplification refractory mutation system (ARMS) method was used according to Baxter et al.[13] Briefly, genomic DNA was isolated from peripheral blood leukocytes according to standard procedures. In a multiplex format, the mutation was detected with the help of allele specific primers (203 bp) and the complete exon 14 was amplified as an internal amplification control (364 bp), taking care not to exceed 0.05 µg of DNA per 50 µl amplification reaction. Amplification products were analyzed after electrophoresis on 4.5% polyacrylamide gels.

Analysis of the MPL W5151K/L mutation

For the detection of MPL mutations in separate reactions, ARMS primers were designed (W515 specific primer GGCCTGCTGCTGCTGAGATG, L515 specific primer GGCCTGCTGCTGCTGAGATT, K515 specific primer GGCCTGCTGCTGCTGAG GAA, and the common primer GGGCGGTATAG TGGGCGTGT) according to Newton et al.[14] with the introduction of extra destabilizing mismatches at position – 2 to increase specificity. Briefly, 10–250 ng of DNA was amplified (pre-cycle: 2 min at 94 °C; 35 cycles: 20 s at 94 °C, 30 s at 64 °C,

30 s at 72 °C; final extension: 2 min at 72 °C) in presence of 1 µM specific and common primers (2 U Taq DNA polymerase, 1.5 mM MgCl2). Amplification products were analyzed by PAGE in 4.5% gels.

Analysis of the JAK2 exon 12 mutation

JAK2 exon 12 mutations were studied as outlined by Scott et al.[15] All patient DNAs were screened by allele specific PCR for K539L, N542-E543del, F537-K539delinsL, and H538QK539L mutations. In addition, the entire exon 12 was sequenced in patients with PV or primary erythrocytosis.

Analysis of the CALR type 1 and type 2 mutations

Analysis of CALR insertions and deletions was performed by fragment analysis essentially as described by Nangalia et al.[16] Samples were tested by amplifying the 3′ part of exon 9 with labeled primers CALRe9F (CCTGCAGGCAGCAGAGAAAC) and CALRe9P (6FAM-ACAGAGACATTATTTGGCGCG). Amplification products were submitted to capillary electrophoresis (ABI 3130, Applied Biosystems de México, SA de CV, Mexico City) and signals were analyzed with the help of GeneMapper ID v3.2 (Applied Biosystems de México, SA de CV, Mexico City).

RESULTS

A total of 27 mestizo individuals with chronic myeloproliferative neoplasias were studied: 14 with ET, eight with PV, four with MF, and one with undifferentiated MPN. Patients with the BCR-ABL1 fusion gene were classified as CML and excluded from the study. Twelve individuals with the JAK2 V617F mutation were found; five of them had been clinically classified as PV, five as ET, one as MF, and one as undifferentiated MPN. One patient with MPL W515L was identified with a clinical picture of ET. Five individuals with the CALR mutation were identified: four with ET and one with MF. On the other hand, of the 14 individuals with ET, five (36%) had the JAK2 V617F mutation, one (7%) had the MPL W515L mutation, and four (29%) had the CALR mutation. Of the eight individuals with PV, five (62%) displayed the JAK2 V617F mutation, whereas of the four patients with MF, one had the JAK2 V617F mutation and one had the CALR mutation. Accordingly, the most consistent relationship was that between PV and the JAK2 V617F mutation (p = .01). Table 1 shows the prevalence of these molecular markers in each category of the Ph1-negative MPN. CALR was the second most frequently mutated gene in MPN, as previously shown.[17] There was no significant difference in gender, age or platelet counts or between patients with mutant CALR and JAK2 V617F. A lower white blood cell count was observed when comparing CALR versus JAK2 V617 mutated and triple negative MPN (p = 0.011). Among all patients, four (14.2%) had a thrombotic episode. JAK2 V617F mutated patients had significantly higher frequencies of thrombosis (p = 0.028).

DISCUSSION

Our understanding of the genetic basis of MPN neoplasms began in 2005, when the JAK2 (V617F) mutation was identified in PV, ET and MF. JAK2 exon 12 and MPL exon 10 mutations were then detected in subsets of patients, and subclonal driver mutations in other genes were found to be associated with disease progression. Recently, somatic mutations

Table 1. *JAK2*, *MPL* and *CALR* mutations in Mexican patients with chronic myeloproliferative neoplasias (MPN). ET = essential thrombocythemia; PV = polycythemia vera; MF = myelofibrosis; uMPN = undifferentiated myeloproliferative neoplasia.

MPN	*n*	*JAK2*		*MPL*		*CALR*	
		V617F	Exon 12 mutations	*W515L*	*W515K*	del52	ins5
ET	14	5	0	1	0	3	1
PV	8	5	0	0	0	0	0
MF	4	1	0	0	0	0	1
uMPN	1	1	0	0	0	0	0
	27	12	0	1	0	3	2

Table 2. *JAK2, MPL* and *CALR* mutations in patients with Philadelphia-chromosome negative chronic myeloproliferative neoplasias (MPN). ET = essential thrombocythemia; PV = polycythemia vera; MF = myelofibrosis; NI: Not investigated; NS: Not specified.

MPN	References	JAK2		MPL		CALR	
		V617F	Exon 12mutations	W515L	W515K	del52 (Type 1)	Ins5 (Type 2)
ET	Tefferi et al.[19]	57%	NS	3%	NI	16%	14%
	Chen et al.[20]	63.9%	NS	2.7%	0%	12.2%	6.1%
	Rumi et al.[21]	62%	0%	4%		11.3%	9.3%
	Quiao et al.[22]	45.5%	NI	0.9%		31.1%	
	Wu et al. [21]	56.3	0%	3.8%	1.3%	25%	
	Akpinar et al.[24]	43.9%	NI	0%		NI	
	This study	36%	0%	7.1%	0%	21%	7%
PV	Quiao et al.[22]	91.9%	2.7%	NI	NI	NS	NS
	Wu et al. [23]	82.5%	12.5%	0%		0%	
	This study	62.5%	0%	0%		0%	0%
MF	Quiao et al. [22]	60.6%	0%	0%		12.1%	
	Xia et al. [25]	50.0%	0%	6.7%	0%	NI	
	Tefferi et al.[19]	54.7%	NI	NI	NI	21%	2.8%
	Akpinar et al. [6]	54.5%	NI	33.3%		NI	NI
	This study	25%	0%	0%		0%	25%

in the gene CALR, encoding calreticulin, have been found in most patients with ET or MF with nonmutated JAK2 and MPL. The JAK-STAT pathway appears to be activated in all MPN, regardless of founding driver mutations. These latter, however, have different effects on clinical course and outcomes. Thus, evaluation of JAK2, MPL, and CALR mutation status is important not only for diagnosis but also for prognostication. These genetic data should now also be considered in designing clinical trials.[17] It has been previously described that:

(a) In PV, the JAK2 (V617F) mutation presents in about 95%, whereas the JAK2 exon 12 mutation presents in 5% of patients.
(b) In ET, the JAK2 (V617F) mutation presents in about 60%, the MPL exon 10 mutations in 5% and the CALR exon 9 mutations in 20% of patients.
(c) In MF, the JAK2 (V617F) mutation presents in about 60%, the MPL exon 10 mutations in 5% and the CALR exon 9 mutations in 20% of patients.

Despite the small size of the study, we have found much less prevalence of JAK2 mutation in PV, ET and MF, which does not match international data The data also confirm our previous observation about the diminished prevalence of PV in Mexico as compared with the prevalence of other MPN in our country.[2,10] As previously described in other parts of the world,[17] in Mexico, the JAK2 (V617F) mutation is the most frequent gene mutation in MPN, followed by the CALR exon 9 mutation, with the MPL and the JAK2 exon 12 mutations being less frequent These data also support the idea that in the study of MPN it is very important to look for the mutation profile of the three genes JAK2, MPL and CALR, since the identification of one of these molecular markers could be considered enough to consolidate a diagnosis of MPN On the other hand, these markers are also useful in the follow-up treatment of some of these diseases, such as the result of bone marrow transplantation in patients with MPNs who display one of these molecular markers, and which can disappear as a result of treatment.[10,18] The Table 2 shows comparative data of our findings with other papers.[19–25]Although we extracted the data from a small-sized patient cohort, this is the first report of CALR mutations in Mexican MPN patients

JAK2 V617F and CALR have been associated with different clinical features in MPN patients. Previous publications reported a higher platelet count

and lower leukocyte and hemoglobin values in CALR mutant patients compared to those with JAK2 V617F or MPL mutations.[21–27] In our cohort, we were not able to find any significant difference for hemoglobin or platelet count, but significantly lower white cell counts for CALR mutated patients were noted ($p = 0.011$). It has been clearly established that JAK2V617F mutation represents a strong independent risk factor for thrombosis and, as previously reported, the incidence of thrombotic events in our study was significantly higher in JAK2 mutated cases when compared to CALR mutated and triple-negative patients ($p = 0.028$).[27,28] Contrary to other studies, we did not find a male predilection or a lower hemoglobin level in CALR-mutated patients, but these inconsistent findings are probably the result of an insufficient case number in our cohort.

Therapeutic uses have been derived from the identification of the molecular markers of MPN. Specific inhibitors of JAK2 have been developed and employed in the treatment of JAK2-mutated MPN.[29] These inhibitors have also been shown to be useful in the treatment of other non JAK2-mutated neoplasias such as other leukemias,[30] and also as anti-cytokines treatment of non-malignant inflammatory conditions[31] and graft versus host disease.[32] It is thus possible that specific inhibitors of MPL or CALR may eventually find a therapeutic use.

A revision of the World Health Organization (WHO) classification of the MPN is ongoing; its classification will hopefully take into account the recent genetic advances in this field. At present, the clinical phenotype of the MPN (ET, PV or MF) continues to be important. Because of their clinical relevance, genetic markers should be properly considered both in clinical decision-making and in the design of clinical trials.[17] In this small study, we found much less prevalence of JAK2 mutation in PV, ET and MF, which does not match international data. Additional studies are needed to either ratify or rectify these findings.

CONFLICT OF INTEREST

The authors state that they do not have conflict of interest.

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