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LETTER TO THE EDITOR

Identification of driver and subclonal mutations in *ASXL1* and *IDH1/IDH2* genes in an Argentine series of patients with myelofibrosis

Myelofibrosis (MF) is a Philadelphia-negative myeloproliferative neoplasm (MPN) characterized by clonal proliferation of hematopoietic stem cells, progressive bone marrow fibrosis, abnormal cytokine expression, anemia, splenomegaly, extramedullary hematopoiesis, constitutional symptoms, cachexia, leukemic progression, and shortened survival.[1] MF can be diagnosed as a primary myelofibrosis (PMF) disorder or as a complication of the evolution of polycythemia vera (MF post-PV) or essential thrombocythemia (MF post-ET).

JAK2, *CALR*, and *MPL* are considered as driver mutations being observed in 90% of patients with PMF. The *JAK2*^{V617F} mutation is the most common with a frequency of ~65%,[1] and its allele burden may be increased by a mitotic recombination phenomenon resulting in the transition from heterozygosity to homozygosity. Lower levels of allele burden were associated with an inferior survival in PMF.[1] Substitutions in *MPL*, mostly affecting codon W515K/L, have been described in ~10% of PMF.[1] *CALR*[1,2] is mainly affected by insertions or deletions (indels) within exon 9 modifying the carboxyl-terminus, giving rise to 2 main variants: type 1, a 52-bp deletion (L367fs*46), or type 2, a 5-bp TTGTC insertion (K385fs*47), which are observed in ~25% of PMF. The remaining 10% of PMF with no driver mutations are considered as "triple negative" (TN) associated with an inferior survival.[3] Recent studies have also identified subclonal mutations affecting genes that encode for epigenetic regulators and members of the splicing machinery. Searching these mutations may help not only in determining the clonal nature of the disease but also in identifying patients with a higher risk. Particularly, those mutations affecting the carboxyl-terminus plant-homeodomain finger of *ASXL1* have been lately associated with poor prognosis.[4] Herein, we have searched for driver (*JAK2*^{V617F}, *MPL*, and *CALR*) and subclonal (*ASXL1*, *IDH1*, and *IDH2*) mutations and described their clinical impact on a series of 39 Argentine patients with MF (28 PMF, 4 MF post-PV, and 7 MF post-ET, according to the WHO criteria). This study was approved by the Institutional Ethical Committee, and written informed consents were obtained from all patients.

In our series of PMF, driver mutations were present with a frequency of 57% (16/28) for *JAK2*^{V617F}, 7% (2/28) for *MPL* W515K/L, and 14% (4/28) for *CALR* [3 cases type 1 (11%)/1 case type 2 (4%)], and 21% (6/28) were TN. The obtained data were in agreement with a recent series of Korean patients with PMF,[5] with a higher frequency of TN than other published series.[1] Those TN patients showed a lower Hb level (Kruskal-Wallis test, $P = .045$) and shorter overall survival (Kaplan-Meier and log-rank test, $P = .009$) (Table 1)

TABLE 1 Hematological profile and clinical characteristics of MF patients according to driver mutations

Variable	*JAK2*^{V617F}	*CALR*	*TN*	*P*
Males (%)	33	50	80	.11[a]
Age (years) (med)	67	67	67	.877[b]
Hemoglobin g/dL (med)	13.1	11.2	7.9	**.045**[b]
WBC 10^9/L (med)	12.8	9.2	7.3	.149[b]
Platelet count 10^9/L (med)	580	527	112.5	.140[b]
Splenomegaly (%)	91	100	100	.58[a]
DIPSS (Int2 + High) (%)	42.9	33.3	57.1	.678[a]
Overall survival (m)	238.3	NR	56.4	**.009**[c]

DIPSS, Dynamic International Prognostic Scoring Systems; WBC, white blood cells; m, months.
Both *MPL*-mutated cases were excluded for the analysis.
[a]Chi-square test.
[b]Kruskal-Wallis test.
[c]Kaplan-Meier and log-rank test, med: median.
Bold indicates statistically significant values.

in coincidence with clinical characteristics already described in TN patients[3] The median *JAK2*^{V617F} allele burdens, measured by an original approach developed by our group,[6] were 67.8% for PMF, 97.0% for MF post-PV and 51.7% for MF post-ET. The higher allele burdens, detected in MF post-PV, confirm previous data,[1] and the small size of our series may be responsible for the lack of statistical significance. (Kruskal-Wallis test, $P = .2586$).

ASXL1 was analyzed by a classical Sanger sequencing approach splitting exon 13 into 2 overlapping fragments. We could identify 5 risk mutations in heterozygous state, including 3 unpublished, and 4 rare missense variants with unknown clinical significance (UCS). Considering both deleterious and UCS variant, we observed a frequency of 28% (11/39) of *ASXL1* mutations in our series (Table 2), being within the reported range for MPN (20%-35%).[4]

Among the detected 5 risk mutations in *ASXL1*, 3 mutations were previously described: the recurrent 22-bp deletion c.1900_1922del (p.E635Rfs*15) that was observed in a patient who developed acute leukemia after 2 years from diagnosis (case 1, Table 2), a missense variant c.3306G>T (p.E1102D) reported in the COSMIC database (http://cancer.sanger.ac.uk/cosmic) with a minor allele frequency

TABLE 2 Characteristics of the PMF patients associated with mutations in *ASXL1*

#	Sex	Age (years)	*ASXL1* mutation	Driver mutation	WBC (cells/mm^3)	Hb (g/dL)	PLTs (#/mm^3)	DIPPS	FU (months)	Therapy	LE	SV
1	M	65	c.1900_1922del (p.Glu635Argfs*15)	*CALR* type 1-like	12 900	10.2	412 000	Int-1(2)	32	Hydroxyurea; JAK 1/2 inhibitor	Yes	Alive
2	M	67	c.3306G>T (p.E1102D)	TN	3200	6.0	35 000	Int-2(4)	23	JAK 1/2 inhibitor	Yes	Dead
3	M	65	c.2187delC (p.Cys730Alafs*14)	*MPL* W515K	28 700	9.6	76 000	Int-2(4)	15	Unrelated donor transplant	No	Dead
4	M	75	c.1921_1928delATCGGAGG (p.Ile641Glyfs*14)	TN	24 000	7.0	54 000	Int-2(4)	16	Thalidomide	No	Alive
5	F	68	c.2984dupA (p.His995Glnfs*2)	*MPL* W515L	66 000	9.7	422 000	High(5)	33	Thalidomide	Yes	Alive
6	M	71	c.1954G>A (p.G652S)	*JAK2*^{V617F}	56 000	7.0	460 000	High(5)	84	HU	Yes	Dead
7	F	66	c.2302C>A (p.Q768K)	CALR type 1-like	12 700	6.9	546 000	Int-2(3)	0	-	No	Alive
8	F	24	c.3692C>T (p.S1231F)	*JAK2*^{V617F}	10 700	13.2	1 110 000	Low(0)	9	-	No	Alive
9	F	73	c.3692C>T (p.S1231F)	*JAK2*^{V617F}	10 400	10.6	1 800 000	Int-1(1)	42	HU	No	Alive
10	F	54	c.3692C>T (p.S1231F)	CALR type 1-like	7200	9.0	312 000	Int-1(1)	54	Epo	No	Alive
11	M	70	c.3745A>G (p.M1249V)	CALR type 2-like	4690	15.5	639 000	Int-1(1)	30	-	No	Alive

Epo, erythropoietin; F, female; FU, follow-up; Hb, hemoglobin levels; HU, hydroxyurea; LE, leukemic evolution; M, male; PLTS, platelet count; SV, survival; TN, triple negative; WBC, white blood cell count; (-) untreated.

Mutations in cases 3, 4, and 5 were not previously reported.

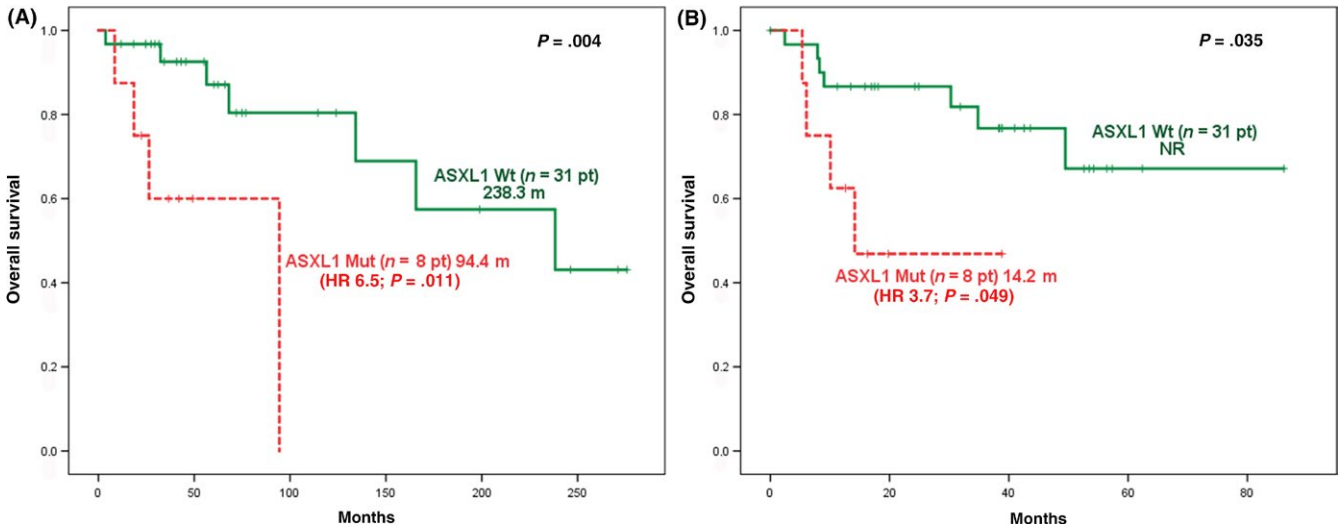

FIGURE 1 Overall survival data among 39 MF patients from the time of (A) diagnosis and (B) from the *ASXL1* testing time to the last follow-up. Patients were stratified by the presence or absence of *ASXL1* mutations. Median survival was assessed by Kaplan-Meier and compared by log-rank method. HR was estimated by Cox regression analysis [Colour figure can be viewed at wileyonlinelibrary.com]

of 0.01, and a deleterious predicted effect by SIFT (http://sift.jcvi.org/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) free available software. This patient suffered from leukemic transformation after 11 months with a fatal outcome associated with a subocclusive episode of intussusception at 9 months (case 2, Table 2). The single nucleotide deletion c.2187delC (p.C730Afs*14), with a predictable frameshift resulting in a premature termination codon (PTC) at codon 743, was detected in a male patient who received an unrelated donor hematopoietic stem cell transplantation with a fatal hepatic veno-occlusive disease at day +100 (case 3, Table 2). Although this mutation has not been indexed to any databases, it has been previously detected in a patient with acute myeloid leukemia. The remaining 2 mutations were *indels* not previously described in the literature or in cancer-related databases (Figure S1): the 8-bp deletion c.1921_1928delATCGGAGG (p.I641Gfs*14) leading to a PTC at codon 654, which was identified in a male patient who presented several poor prognostic factors, such as high age, low hemoglobin levels, low platelet count, peripheral blasts, and red blood cell transfusion dependency (case 4, Table 2); and the single adenosine duplication c.2984dupA (p.H995Qfs*2) generating a PTC at codon 997 that was detected in a female patient who developed acute leukemia 2 years after diagnosis (case 5, Table 2).

Four additional rare missense variants, which are not predicted to have deleterious effects by SIFT and PolyPhen-2 software, were also observed in our series: c.1954G>A (p.G652S), c.2302C>A (p.Q768K), c.3692C>T (p.S1231F), and c.3745A>G (p.M1249V). The variant p.G652S, listed in the dbVar database (https://www.ncbi.nlm.nih.gov/dbvar) under the *rs3746609*, has been detected as a somatic point mutation and is recorded as a benign variant at ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/). This missense variant was observed in a patient who evolved to acute myeloid leukemia (case 6, Table 2). The variant p.Q768K has not been added to any cancer databases nor has been identified in the 1000 Genomes Project database (http://www.internationalgenome.org/). Phylogenetic analyses of orthologous genes indicated that the corresponding *ASXL1* residue Q768 is not conserved among species. The substitution c.3692C>T (p.S1231F) (*rs74638057*) was found in 3 patients (cases 8-10, Table 2) in the context of a rare haplotype *in cis* with the synonym variants c.2985C>T (p.H995=) (*rs62206933*) and c.3519 G>A (p.L1173=) (*rs117901891*). This haplotype was also present in the buccal swab of 1 patient (case 10, Table 2). The fact that the haplotype was present in a tissue of different embryological origin (ectoderm) than hematopoietic cells (mesoderm) confirmed its inherited nature and provided stronger evidence that these changes do not have a deleterious effect per se. This haplotype, however, has been associated with an increased risk of myelodysplastic transformation in a Chinese population with aplastic anemia[7] Therefore, it cannot be entirely ruled out that any of these substitutions, or another variant in linkage disequilibrium with the described haplotype, may have the predisposing effect. The last variant p.M1249V is indexed as *rs146141075*, associated with a minor allele frequency <0.01 with no assessed clinical significance (case 11, Table 2).

Excluding those patients bearing the haplotype based on the evidence that it may be inherited, 21% (8/39) of our series presented variant within the exon 13 of *ASXL1*. These variants conferred a shorter survival either from diagnosis (94 vs 238 months, $P = .004$) or from the testing time (14 months vs not reached, $P = .035$, Kaplan-Meier and log-rank test) (Figure 1). However, we could not confirm its independency as a prognostic marker when the Dynamic International Prognostic Scoring System (DIPSS)[4,8,9] was also considered in the multivariate analyses (Cox regression, enter method), probably associated with the size of the population studied.

IDH1/2 mutations are found in a lower frequency of 5%-7% and have also been considered of prognostic value in PMF[4,8] Therefore, we

have screened their variants by PCR-CSGE (conformation-sensitive gel electrophoresis)[10] and confirmed the abnormal obtained patterns by Sanger sequencing. The screening of *IDH1* and *IDH2* revealed no mutations in our cohort. However, the synonym variant *IDH1* c.315 C>T (p.G105=) could be identified in 4 patients (4/39, 10.2%) in a heterozygous state with a minor allele frequency of 0.051 (4 alleles of 78), similar to the registered in the 1000 Genomes Project database (*rs11554137*). This variant is predicted as pathogenic (score: 0.85) by the FATHMM algorithm (http://fathmm.biocompute.org.uk/). And, it also has been associated with poor prognosis in acute myeloid leukemia,[11] whereas other studies failed to establish this prognostic effect[12] Based on the small number of patients (3 cases with PMF and 1 with MF post-PV) carrying this variation in our series and none of them evolved to acute leukemia during the follow-up, we could not assess the prognostic value of the IDH1 c.315 C>T variant in our MF population.

In conclusion, we present the mutational profile of a series of 39 Argentine patients diagnosed with MF. Driver mutations were observed in very similar frequencies to those published in the Korean population.[5] Regarding *ASXL1*, we have detected 9 rare sequence variants including 2 novels not previously reported in the literature. Excluding 1 haplotype that appears to be constitutional and benign, mutations within exon 13 of *ASXL1* were associated with poor outcome.

This work reinforces the importance of including molecular markers, both drivers and accompanying mutations, to assess the risk of patients with MF. In particular, looking for *ASXL1* mutation allowed identifying patients with an increased risk of premature death, highlighting the importance of these studies to choose the best risk-adapted therapy.

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CONFLICT OF INTEREST

No author has a conflict or duality of interests to report.

AUTHOR CONTRIBUTIONS

IL, CDB, and KS conceived this project. KS, CM, and MR-Z performed the molecular studies. AE, RB, MR-Z, and MMR recruited and evaluated patients studied. KS, YB, and IL managed data, performed statistics, and wrote the manuscript. Each author approved the final version of this manuscript.

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