and more likely to result in viral suppression compared with lamivudine.[8] Retrospective studies have shown that tenofovir may be more effective than entecavir in patients with positive hepatitis B e-antigen, but this was not tested in the 2 patients included in this series.[9] As B-cell aplasia can be prolonged and there are no data at this time on T-cell immune reconstitution after anti-CD19 CAR T-cell therapy, antiviral prophylaxis may need to be continued long-term, as suggested by HBV reactivation experienced by the patient who self-discontinued entecavir 1 year after therapy.

The small sample size does not allow us to determine any association between concomitant HBV or HCV infection and CRS or CRES. Although the etiology of these entities remains to be fully clarified, both seem to be cytokine-driven, with interleukin 6 (IL-6) representing a key molecule.[10] Patients with chronic HBV or HCV infection have higher IL-6 production than healthy controls and all 3 patients discussed herein were treated with anti–IL-6 therapy for CRS/CRES.[11] Future studies will help to clarify the impact of chronic HBV/HCV infection on the risk of CRS and CRES.

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Authorship

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TO THE EDITOR:

Spectrum of *ASXL1* mutations in primary myelofibrosis: prognostic impact of the *ASXL1* p.G646Wfs*12 mutation

Giada Rotunno,[1,2] Carmela Mannarelli,[1,2] Giada Brogi,[1,2] Annalisa Pacilli,[1,2] Francesca Gesullo,[1,2] Francesco Mannelli,[1,2] Sara Fiaccabrino,[1,2] Benedetta Sordi,[1,2] Chiara Paoli,[1,2] Ilaria Marone,[1,2] Elisa Rumi,[3] Rossella Manfredini,[4] Giovanni Barosi,[5] Mario Cazzola,[3] Alessandro M. Vannucchi[1,2,*] and Paola Guglielmelli[1,2,*]

[1]Centro di Ricerca e Innovazione per le Malattie Mieloproliferative (CRIMM), Dipartimento di Medicina Sperimentale e Clinica, Azienda Ospedaliera Universitaria Careggi, and [2]Excellence Centre for Research, Transfer and High Education for the Development of DE NOVO Therapies (DENOTHE), Università degli Studi Firenze, Florence, Italy; [3]Department of Hematology Oncology, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Policlinico San Matteo, University of Pavia, Pavia, Italy; [4]Center for Regenerative Medicine Stefano Ferrari, University of Modena and Reggio Emilia, Modena, Italy; and [5]Center for the Study of Myelofibrosis, IRCCS Policlinico S. Matteo Foundation, Pavia, Italy

The discovery of prognostically informative mutations in patients with primary myelofibrosis (PMF)[1] prompted the development of mutation-enhanced risk scores.[2-6] Among these mutations, those in *ASXL1* are invariably associated with adverse outcome, either as single variant or as part of a high-mutation-risk category (HMR) including *SRSF2*, *EZH2*, and *IDH1/2*.[7,8] A frequently occurring
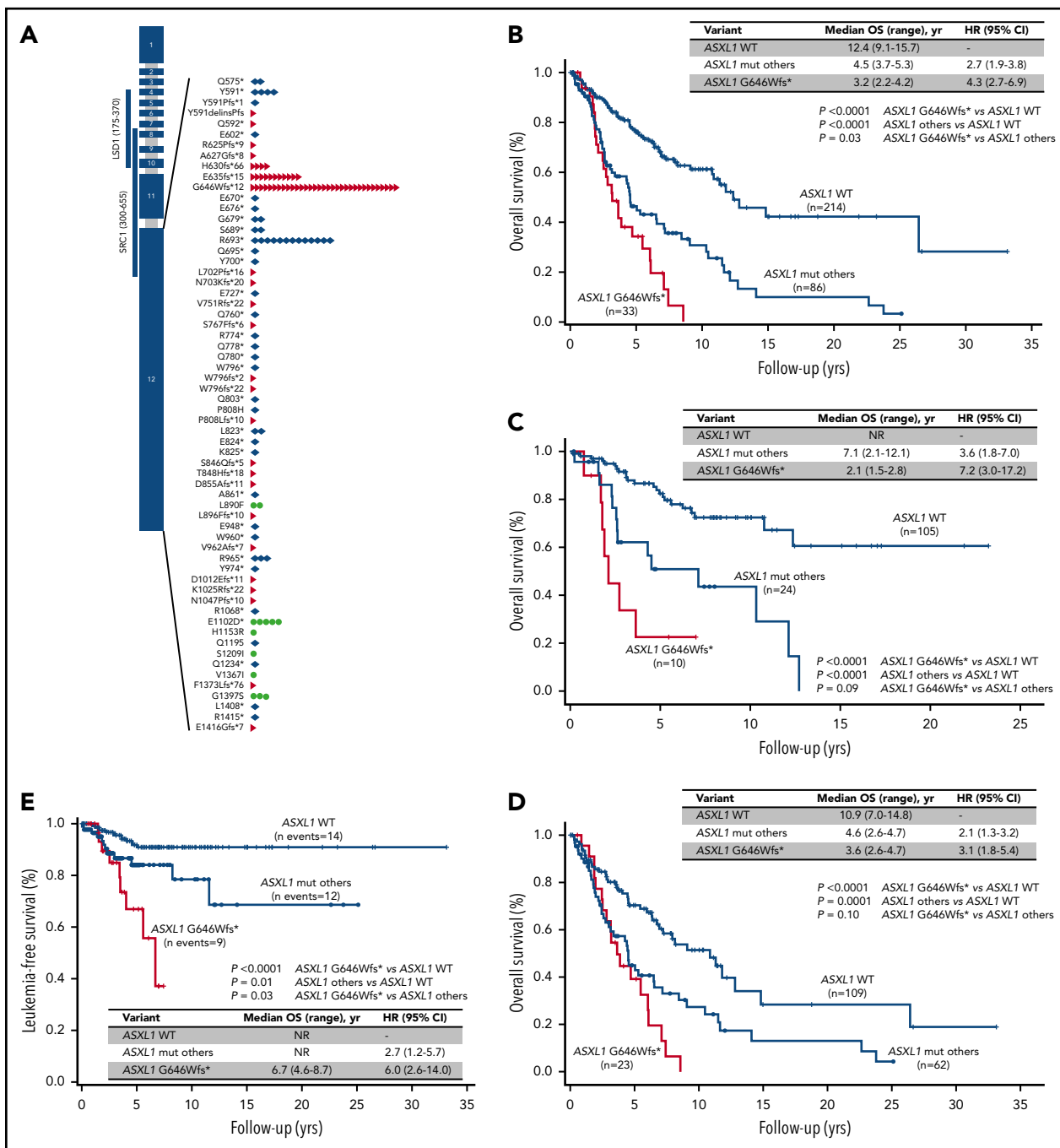
Figure 1. ASXL1 schematic structure and localization of exon 12 mutations. Regions encoding the SRC1-binding domain and lysine-specific demethylase-1 (LSD1)-binding domain are indicated. Each single mutation is shown as: blue diamond, nonsense; red triangle, indel; green dot, missense mutations (A). Overall survival (OS) for patients stratified according to the presence of G646Wfs*12 variant, other ASXL1 mutations, and a wild-type (WT) genotype is shown as Kaplan-Meier survival curves for all patients of the cohort (n = 333; B), patients with prefibrotic PMF (n = 139; C), and overt PMF (n = 194; D). The leukemia-free survival (LFS) for all patients of the cohort is shown in panel E. CI, confidence interval; HR, hazard ratio; mut, mutation; NR, not reached; yr/yrs, year/years.

ASXL1 variant, p.G646Wfs*12 c.1934dup (G646Wfs*12), was long considered an artifact due to sequencing errors owing to the presence of a homopolymer consisting of 8 guanine nucleotides[9]; therefore, this variant may go largely undetected and/or filtered-out in next-generation sequencing (NGS) analysis, possibly resulting in patients' underscoring. To address this point, we evaluated the prognostic impact of ASXL1 G646Wfs*12 in a large population of patients with PMF.

Consecutive cases of PMF, according to 2016 World Health Organization (WHO)[10] criteria, for which an NGS sequence (Personal Genome Machine platform) of the entire ASXL1 exon 12 was available, were recruited from the Centro di Ricerca e Innovazione per le Malattie Mieloproliferative (CRIMM) database (n = 333). The study received institutional review board approval and was conducted in accordance with the Declaration of Helsinki; patients provided written informed consent. Mutation

Table 1. Clinical and laboratory characteristics of study population

Variables	ASXL1 WT, N = 214 n (%) or median (range)	ASXL1 G646Wfsx, N = 33 n (%) or median (range)	ASXL1 other mutations, N = 86 n (%) or median (range)	P ASXL1 WT vs ASXL1 G646W	P ASXL1 WT vs ASXL1 other mutations	P ASXL1 G646W vs ASXL1 other mutations
Follow-up, y	5.6 (0.5-33.1)	3.2 (0.6-8.6)	3.1 (0.2-25.1)	**.004**	**.008**	.57
Prefibrotic PMF diagnosis	105 (49.1)	10 (30.3)	24 (27.9)	**.04**	**.001**	.76
Males	128 (59.8)	25 (75.8)	65 (75.6)	.08	**.01**	.98
Age, y	61.1 (17.7-90.3)	67.4 (48.6-87.3)	63.7 (28.4-89.8)	**.01**	.12	.13
Age >65 y	90 (42.1)	21 (63.6)	36 (41.9)	**.02**	.97	**.03**
Hemoglobin, g/L	12.0 (4.7-16.0)	11.0 (6.2-15.0)	11.5 (5.0-16.5)	.14	.08	.90
Hemoglobin, <100 g/L	48 (22.4)	9 (27.3)	26 (30.2)	.54	.15	.75
Leukocytes, ×10^9/L	8.5 (1.4-106.1)	11.7 (3.5-250.0)	10.7 (1.5-90.8)	**.02**	.10	.39
Leukocytes, >25 × 10^9/L	14 (6.5)	7 (21.2)	17 (19.8)	**.005**	**.001**	.86
Platelets, ×10^9/L	359 (10-1563)	236 (38-1344)	278 (37-1252)	**.04**	.06	.33
Platelets, <100 × 10^9/L	23 (10.7)	6 (18.2)	8 (9.3)	.22	.71	.18
Circulating blasts, ≥2%	13 (6.1)	9 (27.3)	28 (32.6)	**<.0001**	**<.0001**	.56
LDH > UNL, n = 211	122 (90.4)	19 (86.4)	48 (88.9)	.56	.76	.76
Splenomegaly >10 cm from LCM	39 (18.4)	13 (39.4)	29 (34.1)	**.03**	**.003**	.74
BM fibrosis grade				**.05**	**<.0001**	.78
G1	84 (41.4)	8 (26.7)	22 (27.2)			
G2/G3	97 (45.3)	20 (60.6)	56 (65.1)			
Constitutional symptoms	63 (29.4)	17 (51.5)	39 (45.3)	**.01**	**.01**	.55

Bold values in the table body indicate significant correlations at the level of 0.05.

BM, bone marrow; HMR, high-molecular-risk category, points to the presence of any 1 mutation in *ASXL1, EZH2, SRSF2, IDH1/2*; HMR ≥ 2, the presence of 2 or more mutated genes among *ASXL1, EZH2, SRSF2, IDH1/2* (2 or more mutations in the same gene are counted as 1); IPSS, International Prognostic Scoring System; LDH, lactate dehydrogenase; LCM, left costal margin; MIPSS70, mutation-enhanced International Prognostic Scoring System for transplantation-age patients with primary myelofibrosis; MPLW515x, any mutation occurring at *MPL* codon 515; mut, mutation; PMF, primary myelofibrosis; UNL, upper normal limit; WT, wild type.

*Unfavorable karyotype indicates any abnormal karyotype other than normal karyotype or sole abnormalities of 20q−, 13q−, +9, chromosome 1 translocation/duplication, −Y or sex chromosome abnormality other than −Y.

†Triple negative indicates patients who lacked driver mutation.

Table 1. (continued)

Variables	ASXL1 WT, N = 214 n (%) or median (range)	ASXL1 G646Wfsx, N = 33 n (%) or median (range)	ASXL1 other mutations, N = 86 n (%) or median (range)	P ASXL1 WT vs ASXL1 G646W	P ASXL1 WT vs ASXL1 other mutations	P ASXL1 G646W vs ASXL1 other mutations
Karyotype information, n = 264						
Abnormal cytogenetics	45 (29.8)	10 (41.7)	27 (45.8)	.25	**.03**	.73
Unfavorable karyotype*	22 (14.7)	8 (33.3)	18 (30.5)	**.02**	**.009**	.81
IPSS				**<.0001**	**<.0001**	.23
Low	77 (36.0)	2 (6.1)	17 (19.8)			
Intermediate 1	72 (33.6)	11 (33.3)	22 (25.6)			
Intermediate 2	39 (18.2)	7 (21.2)	22 (25.6)			
High	26 (12.1)	13 (39.4)	25 (29.1)			
MIPSS70				**<.0001**	**<.0001**	.85
Low	50 (23.4)	0 (0.0)	0 (0.0)			
Intermediate	92 (43.0)	6 (18.2)	17 (19.8)			
High	72 (33.6)	27 (81.8)	69 (80.2)			
Driver mutation						
JAK2V617F	142 (66.4)	19 (57.6)	52 (60.5)	.27	.35	.68
CALR type1	29 (13.6)	1 (3.0)	13 (15.1)	**.04**	.63	**.04**
CALR type2	12 (5.6)	1 (3.0)	6 (7.0)	.57	.96	.60
MPLW515x	10 (4.7)	2 (6.1)	4 (4.7)	.99	.63	.75
Triple negative†	21 (9.8)	10 (30.3)	11 (12.8)	**.001**	.45	**.02**
Acute leukemia progression	14 (6.5)	9 (27.3)	12 (14.0)	**<.0001**	**.04**	.09
Death	67 (31.3)	26 (78.8)	57 (66.3)	**<.0001**	**<.0001**	.18
HMR	26 (12.1)	33 (100)	86 (100)	**<.0001**	**<.0001**	.99
HMR ≥2	3 (1.4)	19 (57.6)	35 (40.7)	**<.0001**	**<.0001**	.98
EZH2 mut	9 (4.2)	9 (27.3)	17 (19.8)	**<.0001**	**<.0001**	.37

Bold values in the table body indicate significant correlations at the level of 0.05.

BM, bone marrow; HMR, high-molecular-risk category, points to the presence of any 1 mutation in ASXL1, EZH2, SRSF2, IDH1/2; HMR ≥ 2, the presence of 2 or more mutated genes among ASXL1, EZH2, SRSF2, IDH1/2 (2 or more mutations in the same gene are counted as 1); IPSS, International Prognostic Scoring System; LDH, lactate dehydrogenase; LCM, left costal margin; MIPSS70, mutation-enhanced International Prognostic Scoring System for transplantation-age patients with primary myelofibrosis; MPLW515x, any mutation occurring at MPL codon 515; mut, mutation; PMF, primary myelofibrosis; UNL, upper normal limit; WT, wild type.

*Unfavorable karyotype indicates any abnormal karyotype other than normal karyotype or sole abnormalities of 20q−, 13q−, +9, chromosome 1 translocation/duplication, −Y or sex chromosome abnormality other than −Y.

†Triple negative indicates patients who lacked driver mutation.

Table 1. (continued)

Variables	ASXL1 WT, N = 214 n (%) or median (range)	ASXL1 G646Wfsx, N = 33 n (%) or median (range)	ASXL1 other mutations, N = 86 n (%) or median (range)	P ASXL1 WT vs ASXL1 G646W	P ASXL1 WT vs ASXL1 other mutations	P ASXL1 G646W vs ASXL1 other mutations
SRSF2 mut	14 (6.5)	10 (30.3)	16 (18.6)	**<.0001**	**.02**	.17
IDH1/2 mut	6 (2.8)	0 (0.0)	6 (7.0)	.33	.09	.12
CBL mut	3 (1.4)	5 (15.2)	10 (11.6)	**<.0001**	**<.0001**	.65
TET2 mut	39 (18.2)	9 (27.3)	8 (9.3)	.36	**.03**	**.02**
DNMT3A mut	12 (5.6)	1 (3.0)	2 (2.3)	.46	.18	.85
SH3B3 mut	5 (2.3)	0 (0.0)	5 (5.8)	.32	.19	.14
TP53 mut	6 (2.8)	1 (3.0)	6 (7.0)	.92	.15	.38
U2AF1 mut	2 (0.9)	5 (15.2)	7 (8.1)	**<.0001**	**.002**	.31
NRAS mut, n = 233	3 (2.1)	7 (25.9)	9 (14.3)	**<.0001**	**.001**	.19
KRAS mut, n = 233	2 (1.4)	2 (7.7)	4 (6.3)	.05	.06	.80
SF3B1 mut, n = 238	12 (8.2)	0 (0.0)	4 (6.2)	.12	.60	.19
RUNX1 mut, n = 235	3 (2.1)	2 (7.4)	2 (3.1)	.13	.65	.36
cKIT mut, n = 235	1 (0.7)	0 (0.0)	0 (0.0)	.66	.50	

Bold values in the table body indicate significant correlations at the level of 0.05.

BM, bone marrow; HMR, high-molecular-risk category, points to the presence of any 1 mutation in ASXL1, EZH2, SRSF2, IDH1/2; HMR ≥ 2, the presence of 2 or more mutated genes among ASXL1, EZH2, SRSF2, IDH1/2 (2 or more mutations in the same gene are counted as 1); IPSS, International Prognostic Scoring System; LDH, lactate dehydrogenase; LCM, left costal margin; MIPSS70, mutation-enhanced International Prognostic Scoring System for transplantation-age patients with primary myelofibrosis; MPLW515x, any mutation occurring at MPL codon 515; mut, mutation; PMF, primary myelofibrosis; UNL, upper normal limit; WT, wild type.

*Unfavorable karyotype indicates any abnormal karyotype other than normal karyotype or sole abnormalities of 20q−, 13q−, +9, chromosome 1 translocation/duplication, −Y or sex chromosome abnormality other than −Y.

†Triple negative indicates patients who lacked driver mutation.

analysis was performed on purified granulocytes, as described.[2,8,11] All samples were analyzed for ASXL1G646Wfs*12 by bidirectional Sanger sequencing. Survival was calculated from diagnosis to death or last follow-up, or censored at transplantation. The cumulative probability of overall survival (OS) and leukemia-free survival (LFS) was calculated by the Kaplan-Meier method; differences were estimated by the log-rank test. Continuous variables were analyzed by the Mann-Whitney U or Kruskal-Wallis test with the Dunn method for multiple comparison. A $P < .05$ was considered statistically significant.

The list and gene localization of ASXL1 mutations are presented in Figure 1A. ASXL1 nonsynonymous variants were detected in 119 patients (35.7%), including 11 (9.2%) with ≥2 variants. Among these, 72 (56.0%) were frameshift, 49 (38.0%) nonsense, and 8 (6.0%) missense; 45.8% mapped in the steroid receptor coactivator 1 (SRC1)-binding domain and 54.2% in interdomain regions. By NGS, ASXL1G646Wfs*12 was called in 4 patients; after Sanger sequencing, 29 more such cases were identified. Therefore, ASXL1G646Wfs*12 was the most frequent variant, accounting for 27.7% of all ASXL1 mutations, followed by E635fs*15 (9.2%) and R693* (8.4%) (Figure 1A). ASXL1 mutations were enriched in overt PMF compared with pre-PMF (43.8% vs 24.4%; $P = .02$); a similar trend was noted for ASXL1G646Wfs*12 (11.8% vs 7.2%; $P = .07$). All mutations were heterozygous (median [range] variant allele frequency (VAF), 42% [5% to 50%]), except in 1 patient each with Q575* (VAF 71%) and E635fs*15 (VAF, 60%). The VAF of ASXL1G646Wfs*12 (37.5% [9% to 49%]) was similar to other ASXL1 variants (42.0% [5% to 71%]). ASXL1 mutations were more represented in triple-negative patients (50%) compared with patients with JAK2V617F and CALR mutations (33% for both) and MPLW515x mutations (31.5%) ($P = .001$). In particular, ASXL1G646Wfs*12 was found in 47.6% of triple-negative patients compared with JAK2V617F-mutated patients (26.8%), CALR type 1–mutated patients (7.1%), CALR type 2–mutated patients (14.2%), and MPLW515x–mutated patients (16.7%) ($P = .02$; Table 1). Additional nondriver mutations were enriched in ASXL1-mutated patients (71.2%) compared with ASXL1 wild-type patients (52.3%; $P = .001$), with no difference between ASXL1G646Wfs*12 and other ASXL1 variants, other than more TET2 mutations in the former (27.3% vs 9.3%; $P = .02$).

Hematologic and clinical features of the patients, 139 with prefibrotic PMF (41.7%) and 194 overt PMF (58.3%),[10] are shown in Table 1. Median follow-up was 4.5 years (range, 0.1-33.1 years), during which time 150 deaths (45%) and 35 leukemic transformations (10.5%) occurred. The median OS of the entire cohort was 8.1 years (95% confidence interval [CI], 5.9-10.3), 12.4 years (95% CI, 10.3-14.5) for prefibrotic PMF, and 6.5 years (95% CI, 5.2-7.8) for overt PMF ($P = .001$). Compared with the wild-type counterpart, patients harboring any ASXL1 mutation differed for leukocyte, platelet, and blast count; constitutional symptoms; splenomegaly; male sex; diagnosis of overt PMF; bone marrow fibrosis grade ≥ 2; unfavorable karyotype[12]; and more advanced International Prognostic Scoring System (IPSS) and mutation-enhanced International Prognostic Scoring System for transplantation-age patients with PMF (MIPSS70) categories (Table 1). No notable difference was found by comparing ASXL1G646Wfs*12 and other ASXL1 mutations.

Of the 150 patients who died, 78.8%, 66.3%, and 31.3% had ASXL1G646Wfs*12, ASXL1 others, and wild-type genotype,

respectively ($P < .0001$ vs wild-type patients). There was no differential impact on survival of ASXL1 mutation type (ie, frameshift, including ASXL1G646Wfs*12, nonsense and missense), whereas each of them conferred adverse prognosis compared with wild-type genotype (supplemental Figure 1, available on the Blood Web site; $P < .0001$ for all), confirming the report of others.[13] In particular, the median (range) survival of ASXL1G646Wfs*12-mutated patients was 3.2 years (2.2-4.2 years), significantly shorter than other ASXL1 mutations (4.5 years [3.7-5.3 years]; $P = .03$) and wild-type genotype (12.4 years [9.1-15.7 years]; $P < .0001$) (Figure 1B). The hazard ratio (HR) (95% CI) for survival vs wild-type patients was 4.3 (95% CI, 2.7-6.9) and 2.7 (95% CI, 1.9-3.8) for ASXL1G646Wfs*12 and other ASXL1 mutations, respectively.

The adverse impact of ASXL1G646Wfs*12 was also confirmed by separate analysis of pre-PMF and overt PMF patients (Figure 1C-D). Median (range) survival of patients harboring ASXL1G646Wfs*12 was 2.1 years (1.5-2.8 years) and 3.6 years (2.6-4.7 years) in pre-PMF and overt PMF, respectively, compared with 7.1 years (2.1-12.1 years) and 4.6 years (2.6-4.7 years) for other ASXL1 mutations, and not reached and 10.9 years (7.0-14.8 years) for wild-type patients ($P < .0001$), respectively. The HR for ASXL1G646Wfs*12 and other ASXL1 mutations was 7.2 (95% CI, 3.0-17.2) and 3.6 (95% CI, 1.8-7.0) in pre-PMF, and 3.1 (95% CI, 1.8-5.4) and 2.1 (95% CI, 1.3-2.1) in overt PMF ($P < .0001$). We then performed multivariable analysis to validate the prognostic significance of ASXL1G646Wfs*12 in the context of MIPSS70 and MIPSS70plusV2.0. Results indicate that ASXL1G646Wfs*12 remained independently associated with shortened survival, as well as any other ASXL1 mutation (supplemental Table 1).

These findings were validated in an independent cohort of 271 patients, 81% with overt PMF, collected in 2 other institutions. NGS analysis identified ASXL1 mutations in 70 patients (25.8%) including ASXL1G646Wfs*12 in 2 (2.8%). However, after Sanger sequencing, ASXL1G646Wfs*12 was detected in 20 additional cases (7.4%), to a total of 90 ASXL1-mutated patients (33.2%); ASXL1G646Wfs*12 was the most frequent variant (24.4% of total). Of the 144 patients who died (53.1%), 43.7% harbored ASXL1 mutations, and the proportion of those with ASXL1G646Wfs*12 was 31.2% ($P < .0001$ vs ASXL1 others). The median (range) survival of patients harboring ASXL1G646Wfs*12 was 3.8 years (0.5-5.5 years), compared with 5.9 years (4.0-7.7 years) ($P = .04$) for other ASXL1 mutations and 8.4 years (7.0-9.7 years) for wild-type genotype ($P < .0001$) (supplemental Figure 2).

Thirty-five patients transformed to leukemia (10.5%), of whom 27.3%, 14.0%, and 6.5% had ASXL1G646Wfs*12, other ASXL1 mutations, and wild-type genotype, respectively ($P < .0001$). The LFS was significantly shorter in patients with ASXL1G646Wfs*12 (6.7 years; range, 4.6-8.7 years) compared with ASXL1 others (not reached; $P = .03$) and wild-type patients (not reached; $P < .0001$) (Figure 1E). The HR (95% CI) vs wild-type patients was 6.0 (95% CI, 2.6-14.0) and 2.7 (95% CI, 1.2-5.7) for ASXL1G646Wfs*12 and other ASXL1 mutations, respectively. Due to the low number of events in the validation cohort, attempts to validate these findings were not performed.

In summary, data reported herein suggest that care should be taken to accurately detect and report the ASXL1G646Wfs*12

variant in patients with PMF because of its strong adverse impact on OS and LFS. A recent report provided extensive bioinformatic advice to correctly call this variant from background noise in NGS traces, concluding that *ASXL1*G646Wfs*12 variant is a bona fide mutation[14]; another study indicated that this variant may represent an early mutational event in a dominant clonal population of hematopoietic cells.[15] Finally, it seems reasonable, but remains to be shown, that *ASXL1* G646Wfs*12 has similar adverse prognostic relevance in myelodysplastic syndromes and acute leukemia.

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Authorship

Contribution: G.R. performed the research and contributed to data analysis and manuscript writing; G. Brogi, C.M., S.F., F.G., R.M., and A.P. performed the research and contributed to data analysis; F.M., B.S., C.P., E.R., G. Barosi, M.C., and I.M. provided patient samples and clinical information; and A.M.V. and P.G. designed the research, analyzed the data, and wrote the manuscript.

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ORCID profiles: F.M., 0000-0003-4810-6501; E.R., 0000-0002-7572-9504; G.B., 0000-0002-8357-7192; A.M.V., 0000-0001-5755-0730; P.G., 0000-0003-1809-284X.

Correspondence: Alessandro M. Vannucchi, AOU Careggi, Dipartimento di Medicina Sperimentale e Clinica, Centro di Ricerca e Innovazione, e Laboratorio Congiunto, per le Malattie Mieloproliferative, Viale Pieraccini, 6, 50134 Florence, Italy; e-mail: amvannucchi@unifi.it.

Footnotes

*A.M.V. and P.G. contributed equally to this work.

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