Disruption of the *ASXL1* gene is frequent in primary, post-essential thrombocytosis and post-polycythemia vera myelofibrosis, but not essential thrombocytosis or polycythemia vera: analysis of molecular genetics and clinical phenotypes

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ABSTRACT

Background
The myeloproliferative neoplasms, essential thrombocytosis, polycythemia vera and primary myelofibrosis, share the same acquired genetic lesion, but the concept of *JAK2* V617F serving as the sole lesion responsible for these neoplasms is under question, and there has been interest in identifying additional mutations that may contribute to disease pathogenesis. Because *ASXL1* lesions have been increasingly identified in myeloid neoplasms, we examined the relationships of *ASXL1* mutation or deletion to both clinical phenotype and associated molecular features in 166 patients with myeloproliferative neoplasms.

Design and Methods
Exon 12 of *ASXL1* was amplified from neutrophil genomic DNA and bidirectionally sequenced in 77 patients with myelofibrosis (including patients with primary and post-essential thrombocytosis or post-polycythemia myelofibrosis), 42 patients with polycythemia vera, 41 with essential thrombocytosis and 6 with post-myelofibrosis acute myeloid leukemia. Pyrosequencing assays were designed to determine the allele percentages of *JAK2* V617F (G5073770T), *ASXL1* 2475dupA, and *ASXL1* 2846_2847del in neutrophil genomic DNA samples. Clinical and laboratory characteristics of patients with wild-type and *ASXL1* mutations were then compared.

Results
We identified nonsense mutations or hemizygous deletion of *ASXL1* in 36% of the patients with myelofibrosis, but very rarely among those with polycythemia vera or essential thrombocytosis. Among the patients with myelofibrosis, those with *ASXL1* lesions were not distinguished from their wild-type counterparts with regard to *JAK2* V617F status, exposure to chemotherapy or evolution to leukemia. Myelofibrosis patients with *ASXL1* lesions were more likely to have received anemia-directed therapy compared to those without lesions [15/26 (58%) *versus* 11/39 (23%); P=0.02]. Using serial banked samples and quantitative *ASXL1* mutant allele burden assays, we observed the acquisition and accumulation of *ASXL1* mutations over time in two patients with post-essential thrombocytosis myelofibrosis.

Conclusions
ASXL1 haploinsufficiency is associated with a myelofibrosis phenotype in the context of other known and unknown lesions, and disruption of *ASXL1* function may contribute to the disease pathogenesis of myelofibrosis.

Key words: *ASXLI*, haploinsufficiency, myelofibrosis, pathogenesis.

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Introduction

The myeloproliferative neoplasms (MPN), essential thrombocytosis (ET), polycythemia vera (PV), and primary myelofibrosis (PMF) share the same acquired genetic lesion, *JAK2* V617F, but differ with respect to epidemiology and natural history. PMF is the rarest of the classical MPN, occurs predominantly in males, and manifests at an older age compared to ET and PV.[1,2] PMF carries the worst prognosis because of complications of bone marrow failure or leukemic transformation. These phenotypic differences raise the question of how a shared genetic lesion could result in three distinct disorders.

Within the *JAK2* V617F-positive MPN, variation in both gene dosage of *JAK2* V617F and the disease burden at the progenitor level offer some rationale for the discrepancy between genotype and phenotype. Low *JAK2* V617F allele burdens associate with ET and higher allele burdens associate with PV, PMF, post-ET myelofibrosis (post-ET-MF) and post-PV myelofibrosis (post-PV-MF); however, significant overlap in allele burdens across these groups occurs. In the majority of cases of myelofibrosis, the neoplastic clone seems to be dominant at the expense of wild-type precursors, indicating a dominant biology of the disease MPN clone at the stem cell level.[3-5] These clinical and molecular distinctions may reflect an intrinsic genetic complexity unique to primary and secondary myelofibrosis compared to ET or PV.

The intrinsic genetic complexity in myelofibrosis may have its basis in the acquisition of additional genomic lesions independent of *JAK2* V617F, since 50% of cases are negative for the mutation. Acquired somatic mutations of the *ASXL1* gene have recently been shown to be prevalent in many myeloid disorders, including acute myelogenous leukemia (AML), chronic myelomonocytic leukemia, and chronic myelogenous leukemia and myelodysplastic syndromes.[6-10] We examined the relationships of *ASXL1* mutation or deletion to both clinical phenotype and associated molecular features in 166 MPN patients using both cross-sectional and longitudinal analyses.

Design and Methods

Patients

The study population consisted of 166 patients with MPN (ET, PV, PMF, post-ET-MF, and post-PV-MF) and AML arising from antecedent myelofibrosis evaluated in the Johns Hopkins Center for Chronic Myeloproliferative Disorders between May 2005 and January 2011 (Table 1). The diagnosis of PV and ET was based on the Polycythemia Vera Study Group criteria[11,12] or World Health Organization (WHO) criteria[13,14] depending on the year of diagnosis; the diagnosis of PMF was based on Italian Consensus criteria[15] or WHO criteria[13] depending on the year of diagnosis. The study was approved by the Institutional Review Board and all patients gave written consent to participation.

Genomic analysis

DNA from neutrophils purified from peripheral blood samples was prepared as previously described.[16] Neutrophils were selected as the representative tumor tissue due to the observations that in the MPN, clonal dominance of the tumor is present at the neutrophil level, while polyclonal hematopoiesis may persist at the marrow progenitor level potentially diluting tumor DNA with DNA from residual wild-type cells.[3,4] The *JAK2* V617F neutrophil allele burdens were measured using an allele-specific, quantitative real-time polymerase chain reaction (PCR) assay sensitive to a lower limit of detection of 5% of either the wild-type or mutant *JAK2* allele as previously described.[4] Single nucleotide array karyotyping assays and analyses were performed as described previously.[17-19] Briefly, Gene Chip Mapping Affymetrix 250K arrays (Affymetrix, Santa Clara, CA, USA) were used for single nucleotide polymorphism karyotyping according to the manufacturer's instructions. Signal intensity was analyzed and single nucleotide polymorphism calls determined using Gene Chip Genotyping Analysis Software Version 4.0 (GTYPE). Copy number and areas of uniparental disomy were investigated using a Hidden Markov Model and Copy Number Analyzer for Affymetrix GeneChip Mapping 250K arrays (CNAG v3.0).

Direct polymerase chain reaction sequencing

Exon 12 of *ASXL1* was amplified from neutrophil genomic DNA using PCR primers described by Gelsi-Boyer *et al.*[7] PCR amplifications were done in a total volume of 25 μL using the

Table 1. Characteristics of the 166 individuals forming the MPN cohort.

Diagnosis	N.	% Female	Median age (range)*	Median disease duration (years, range)**	*JAK2* V617F positive cases (%)	Median JAK2V617F allele burden in JAK2V617F + cases	*ASXL1* nonsense mutation (n, %)	*ASXL1* hemizygous deletion (n, %)	Total *ASXL1* mutant or deleted (%)
ET	41	68	55 (20-86)	6 (0-30)	44	38	0(0%)	0	0
PV	42	62	57 (15-78)	6 (0-29)	88 ****	61	1(2%)	0	1(2%)
All MF	77	43	65 (44-90)	6 (0-37)	70	59	23(30%)	5 (6.4%)	28 (36%)
Post-ET-MF	16	50	61 (51-85)	12 (5-31)	43	50.5	6 (38.5%)	2 (12.5%)	8 (50%)
Post-PV-MF	14	43	63 (51-78)	14(2-37)	100	90	5 (36%)	0	5 (36%)
PMF	47	40	66 (44-90)	2 (0-26)	69	57	12 (26%)	3 (6%)	15 (32%)
AML***	6	50	67 (51-84)	5.5 (1-11)	67	61	2 (33%)	0	2 (33%)
Total	166	54					26(16%)	5 (3%)	31 (19%)

**Median age refers to age at sample analysis. **Median disease duration refers to the number of years from the onset of the MPN at the time of sample analysis. ***These patients had AML at the time of ASXL1 sample analysis, five of whom had antecedent myelofibrosis (MF) and one of whom had antecedent PV. ****Includes four PV cases with characterized JAK2 exon 12 mutations, and one PV patient who is mutation-negative.*

Qiagen Taq PCR Master Mix kit (Qiagen, Valencia, CA, USA) and following the manufacturer's procedures. PCR amplification conditions were as follows: 50°C for 2 min; 94°C for 3 min; 94°C for 1 min, 56°C for 1 min, and 72°C for 1.5 min for 35 cycles; then 72°C for 10 min. Aliquots of purified PCR products were sequenced at the JHU DNA Analysis Facility using an Applied Biosystems 3730xl DNA Analyzer. All sequencing reactions were performed bidirectionally, and all sequence variation positive reactions were repeated. Sequence analysis included direct examination of the electropherograms and alignment of sequences compared to *ASXL1* genomic DNA in GenBank (NM 015338.5) using the ClustalW2 multiple sequence alignment program (*www.ebi.ac.uk/Tools/msa/clustalw2/*). Known single nucleotide polymorphisms were identified by searching the NCBI dbSNP database (*www.ncbi.nlm.nih.gov/snp*) and the 1000 Genomes Project (*www.1000genomes.org*).

ASXL1 mutant allele quantitation by pyrosequencing

We used Pyromark Assay Design 2.0 software (Qiagen) to design pyrosequencing assays to determine the allele percentages of *JAK2* V617F (G5073770T),[20] *ASXL1* 2475dupA, and *ASXL1* 2846_2847del in samples of neutrophil genomic DNA (*Online Supplementary Table S1*). PCR amplification conditions for all primer sets were as follows: 50°C for 2 min; 94°C for 3 min; 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min for 35 cycles; then 72°C for 10 min. Ten microliters of the PCR reaction were subjected to analysis with PyroMark Q24 pyrosequencer (Qiagen), using reagents supplied by the manufacturer. The percentages of *ASXL1* mutant alleles were determined using the Allele Frequency Quantification function in the PyroMark Q24 Software 2.0 package according to the manufacturer's specifications (Qiagen).

Genotype-phenotype correlations

Age at sampling and diagnosis, disease duration, spleen size (measured in centimeters below the costal margin), blood cell counts, treatment status (hydroxyurea, or anemia-directed therapy, including transfusions, erythropoietin, steroids, and immunomodulatory agents such as thalidomide), *JAK2* status and mutational burden if applicable, and single nucleotide polymorphism karyotyping results (presence of uniparental disomy or chromosomal losses/gains) were assessed at the time of *ASXL1* sequencing analysis.

Statistical analysis

Fisher's exact test and χ^2 testing were used to detect significant differences in categorical variables. Testing for differences in mean proportions was performed when appropriate with z tests. The non-parametric Wilcoxon's rank sum test was used to detect significant differences in the median values of continuous variables. A two-tailed *P* value of 0.05 or less was considered statistically significant. Data were analyzed using the STATA SE 10.1 program (STATA Corp, College Station, TX, USA).

Results

ASXL1 sequence variation in the patients with myeloproliferative neoplasms

We sequenced *ASXL1* exon 12 in 77 patients with myelofibrosis, 42 with PV, 41 with ET and 6 with post-myelofibrosis AML (Table 1). *JAK2* V617F status and quantitative *JAK2* V617F neutrophil allele burdens were determined for all patients and are presented in Table 1, including those for five PV patients who were *JAK2* V617F-negative, four of whom harbor *JAK2* exon 12 mutations, while the fifth could not be molecularly defined. We found 28 nonsense lesions consisting of insertions, deletions or single nucleotide substitutions inducing stop lesions in 23 of the patients with myelofibrosis, in one with PV but in none of the ET patients (Tables 1 and 2). In four of the *ASXL1*-mutation-positive cases of myelofibrosis (UPIN numbers 143, 376, 453 and 1327), more than one distinct *ASXL1* mutation was identified (Table 2). The distribution of the mutations across *ASXL1* in this cohort of patients with MPN was similar to that reported in the Catalogue of Somatic Mutations in Cancer (*http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=gene&ln=ASXL1*) for a variety of hematologic diseases and in a large AML *ASXL1* sequencing project[6] with the majority of lesions involving amino acids 591-888 (Table 2). Two

Table 2. *ASXL1* nonsense sequence variation and deletion in the MPN cohort.

UPIN	**Age/Sex	Diagnosis	Nucleotide	Amino Acid
1221	68/M	PMF/AML	1772dupA	Y591X
376*	49/M	PMF	1900_1922del	E635RfsX15
1292	75/M	PMF	1900_1922del	E635RfsX15
568	74/F	PMF	1915_1928del	T639GfsX14
114	48/F	PV	1934dupG	G646WfsX12
143*	52/M	PMF	1934dupG	G646WfsX12
376*	49/M	PMF	1934dupG	G646WfsX12
453*	60/M	PET-MF	1934dupG	G646WfsX12
1274	66/M	PMF	1934dupG	G646WfsX12
1314	76/M	PMF	1934dupG	G646WfsX12
1327*	62/M	PET-MF	1934dupG	G646WfsX12
1330	51/F	PMF	1934dupG	G646WfsX12
1373	64/F	PMF	1934dupG	G646WfsX12
1471	65/M	PMF	1934dupG	G646WfsX12
1214	64/F	PPV-MF	1963_1976del	T655WfsX8
1087	54/M	PPV-MF	2061delT	C687Xfs
1095	73/M	PPV-MF to AML	2118_2119del	T707PfsX10
1400	75/F	PMF	2190C >A	C730X
1486	64/F	PMF	2278C >T	Q760X
1299	48/F	PMF	2290delC	L764YfsX8
1382	76/M	PMF	2429delA	D180AfsX8
1327*	62/M	PET-MF	2428_2440del	D810FfsX4
143*	52/M	PMF	2464dupA	T822NfsX10
488	74/F	PET-MF	2475dupA	G826RfsX6
1182	72/M	PPV-MF	2507delC	T836IfsX2
1431	61/M	PMF	2485C >T	Q829X
453*	60/M	PET-MF	2846_2847del	G949AfsX5
561	64/F	PET-MF	2898_2900del	G967del
1463	53/F	PPV-MF	3005_3135dup	V1046PfsX22
1388	44/M	PMF	4139_4140del	A1380EfsX11
800	53/M	PET-MF	Hemizygous deletion of *ASXL1*	
1290	65/M	PMF	Hemizygous deletion of *ASXL1*	
1319	65/F	PMF	Hemizygous deletion of *ASXL1*	
1348	67/M	PMF	Hemizygous deletion of *ASXL1*	
1441	85/F	PET-MF	Hemizygous deletion of *ASXL1*	

**Indicates the four individuals in whom more than one ASXL1 lesion was detected. **Age at the time of sample analysis. PET: post-ET; PPV: post-PV.*

Table 3. *ASXL1* missense sequence variations in the MPN cohort.

UPIN	Age/Sex	Diagnosis	Nucleotide	Amino Acid	dbSNP or 1000 Genomes Project
1032	79/M	ET	1954G >A	G652S	rs3746609
1130	41/M	ET	2395G >T	D799Y	not reported
383	65/M	PMF	2400T >G	D800E	not reported
1338	58/F	ET	2513A >G	K838R	rs35632616
1334	67/M	PMF	3306G >T	E1102D	not reported
356	58/F	PMF	3935C >T	A1312V	not reported
1359	26/F	ET	4399 C >T	L1325F	rs6057581

recurrent lesions, 1934dupG and 1900_1922del, present in 38% (10/26) and 8% (2/26), respectively, of the mutation-positive MPN patients, were also the two most common lesions in the AML cohort,[6] occurring in 66% and 7% of those patients, respectively. We found seven missense lesions in seven MPN patients, only three of which were reported as single nucleotide polymorphisms in dbSNP or the 1000 Genomes Project (r3746609, rs35632616 and rs6057581, Table 3).

ASXL1 *hemizygous deletion identified by single nucleotide polymorphism karyotyping*

We performed single nucleotide polymorphism array karyotyping on neutrophil genomic DNA from all of the ET and PV patients (n=41 and 42, respectively), and from 67 of the 77 patients with myelofibrosis from the *ASXL1* sequencing project (DNA from the other 10 patients with myelofibrosis was insufficient for the analysis). Detailed results of this analysis are to be presented elsewhere (McDevitt, *unpublished data*). 20q11deletions were identified in 7/67 (10.4%) of the cases of myelofibrosis and in none of the PV or ET cases. The deletion region involved only one copy of chromosome 20q, as determined by both metaphase cytogenetics and single nucleotide polymorphism karyotyping analysis in all cases, and in five of these cases, the deletion region spanned bases 30,158,806_34,151,223, encompassing 73 genes including *ASXL1* (chromosome 20q11: 30946147_31027122) (*Online Supplementary Figure S1*). Of the five cases of myelofibrosis with the 20q11 deletion inclusive of *ASXL1*, three were *de novo* and two were post-ET-MF (Tables 1 and 2). The 20q deletion region was not otherwise involved by uniparental disomy or chromosomal gains in either the cases of myelofibrosis or the rest of the MPN cohort who underwent single nucleotide polymorphism karyotyping. All cases of myelofibrosis in which the *ASXL1* gene was affected by hemizygous deletion were resequenced and in none of these was the remaining *ASXL1* allele affected by a nonsense lesion (Table 2).

ASXL1 *genotype/phenotype correlations in myelofibrosis*

We collected the clinical and molecular features of 73 of the 77 patients with myelofibrosis in whom *ASXL1* status was determined by direct sequencing and single nucleotide polymorphism karyotyping. Four patients with myelofibrosis were censored from this analysis because of incomplete clinical information (n=1) or because they had missense lesions that are not reported as single nucleotide polymorphisms, whose effect on ASXL1 function is unknown (n=3) (Table 3) and due to the fact that suitable germline tissue to determine the somatic nature of these sequence variations was not available. We, therefore, compared 28 *ASXL1* lesion-positive myelofibrosis patients (23 cases with *ASXL1* nonsense lesions and 5 cases with *ASXL1* hemizygous deletions, Table 2) to 45 myelofibrosis patients with wild-type *ASXL1* (Table 4). *ASXL1* deletion or mutation was equally prevalent in PMF (15/45, 33%), post-PV-MF (5/14, 36%) and post-ET-MF (8/16, 50%) (Table 4). *ASXL1* lesions were not associated with exposure to chemotherapy as the prevalence of hydroxyurea use was similar in patients with and without lesions, and *ASXL1* lesions were present in patients who had never received any form of chemotherapy (Table 4). There was no dependence upon *JAK2* status as the presence of *ASXL1* lesions was identified in *JAK2* V617F-negative cases (9/28) (one patient of whom harbored a *JAK2* exon 12 lesion, H538K539delinsL, UPIN 1182, Table 2), *JAK2* V617F-heterozygous cases (9/28), and *JAK2* V617F-homozygous cases (10/28). Based on the results of single

Table 4. Characteristics of the patients with myelofibrosis divided by *ASXL1* status.

	ASXL1 wild-type (n=45)*	*ASXL1* mutant (n=28)	*P* value
Diagnosis, n. (%)			
All			0.49
PMF	29 (64)	15 (54)	0.27
Post-ET-MF	8 (18)	8 (28)	0.31
Post-PV-MF	8 (18)	5 (18)	0.83
Male sex, n (%)	26 (58)	16 (57)	0.99
Age at MPN diagnosis (years), median (IQR)	59 (51, 65)	55 (42, 65)	0.14
Age at MF diagnosis (years), median (IQR)	62 (54, 69)	61 (53, 68)	0.95
Age at sampling (years), median (IQR)	66 (59, 72)	64 (56, 73)	0.67
MPN disease duration (years), median (IQR)	6 (1, 11)	10 (2, 23)	0.11
MF disease duration (years), median (IQR)	1 (1, 5)	2 (1, 5)	0.97
Transition from ET to MF (years), median (IQR)	9 (5, 13)	17 (10, 24)	0.08
Transition from PV to MF (years) median (IQR)	9 (5, 14)	20 (10, 35)	0.16
Spleen size, cm below costal margin (IQR)	6 (1, 14)	11 (2, 14)	0.26
White blood cell count ($\times 10^9$/L)	12.5 (5.5, 21.7)	10.7 (5.5, 18.6)	0.61
Hemoglobin (g/dL)	11 (9.3, 11.9)	9.9 (9, 11.7)	0.25
Platelet count ($\times 10^9$/L)	232 (137, 477)	172 (120, 335)	0.45
Reticulocyte count ($\times 10^9$/L)	84.4 (63.7, 115)	92 (50.7, 146.3)	0.70
Hydroxyurea, n. (%)	13 (29)	10 (36)	0.61
Anemia therapy, n. (%)	11 (24)	15 (54)	0.023
JAK2 V617F status, n. (%)			
All			0.53
Negative	13 (29)	9** (32)	0.79
JAK2 V617F neutrophil allele burden, % (*ASXL1* wild-type=32) (*ASXL1* mutant=19)	61 (51, 83)	57 (49, 90)	0.47
Associated UPD, n. (%) (*ASXL1* wild-type=38) (*ASXL1* mutant=25)	19 (50)	13 (52)	0.99
Associated loss (*excluding 20q deletion*)/gain, n. (%) (*ASXL1* wild-type=38) (*ASXL1* mutant=25)	17 (45)	12 (48)	0.99

*four patients from the 77 MF patients listed in Table 1 were censored from the analysis, one with post-PV-MF due to lack of clinical information and three with PMF (listed in Table 3) whose ASXL1 missense lesions could not be verified as wild-type due to lack of germline control tissue. **Includes one PV patient with JAK2 H538K539delinsL; UPD: uniparental disomy.

nucleotide polymorphism karyotyping, patients with *ASXL1* lesions were equally as likely to have uniparental disomy (involving 9p or other regions), or chromosomal loss or gain abnormalities compared to those without *ASXL1* lesions (Table 4). There were no differences in sex, age, or disease duration between myelofibrosis patients with and without *ASXL1* lesions. Median white blood cell counts (10.7×10^9/L *versus* 12.5×10^9/L; P=0.61), hemoglobin concentration (9.9 *versus* 11 g/dL; P=0.25), and platelet counts (172×10^9/L *versus* 232×10^9/L; P=0.45) did not differ between myelofibrosis patients with and without *ASXL1* lesions. However, myelofibrosis patients with *ASXL1* lesions were more likely to have received anemia-directed therapy (transfusion, erythropoietin, immunomodulatory agents such as thalidomide and steroids) compared to those without lesions [15/28 (54%) *versus* 11/45 (24%); P=0.023] (Table 4).

The patients with post-ET-MF comprised 28% (8/28) of the *ASXL1*-lesion positive cases, compared to only 13% (6/45) of the *ASXL1*-wild type cases (P=0.11). Moreover, *ASXL1* lesions were most prevalent in the post-ET-MF group, being present in 8/16 individuals (Table 1). However, the presence of an *ASXL1* lesion was not associated with an accelerated transition rate from ET or PV to myelofibrosis. In fact, the median time of transition from ET or PV to myelofibrosis was longer in those with *ASXL1* lesions than in those without *ASXL1* lesions (17 *versus* 9 years, respectively in ET to myelofibrosis, P=0.08; 20 *versus* 9 years in PV to myelofibrosis, P=0.16) but these differences were not statistically significant.

Six patients had AML at the time of the *ASXL1* analysis and two of these six (33%) patients were found to have *ASXL1* nonsense lesions (Tables 1 and 2). Eleven patients from the remaining 160 MPN patients from this cohort (Table 1) subsequently developed AML, eight of whom were male, ten of whom had antecedent myelofibrosis and one of whom had PV (Table 5). *ASXL1* nonsense lesions were detected in the MPN phase of 4/10 of the PMF cases from this group, which was not different from the prevalence of *ASXL1* nonsense lesions in the myelofibrosis cohort as a whole or the AML cohort (23/77 and 2/6, respectively, Table 1). *ASXL1* lesions were evident in the latent MPN phase as long as 9 years before the development of AML (Table 5). Unfortunately, purified

Table 5. Features of MPN patients who developed AML.

UPIN	*Age /Sex	MPN	Neutrophil JAK2 V617F %	*ASXL1* lesion	Disease year at *ASXL1* analysis	Disease year at AML diagnosis
136	84/F	PV	98	no	14	14
143	60/M	PMF	75	G646WfsX12; T822NfsX10	2	11
488	75/F	PET-MF	0	G826RfsX6	9	15
544	74/M	PMF	89	no	15	16
1095	73/M	PPV-MF	70	T707PfsX10	3	5
1221	68/M	PMF	52	Y591X	11	11
1243	63/M	PPV-MF	93	no	6	7
1361	66/M	PMF	0	no	3	3
1413	64/F	PMF	54	no	5	6
1424	58/M	PMF	0	no	1	2
1444	65/M	PMF	0	no	1	2

**Age at time of AML diagnosis; PET: post-ET; PPV: post-PV.*

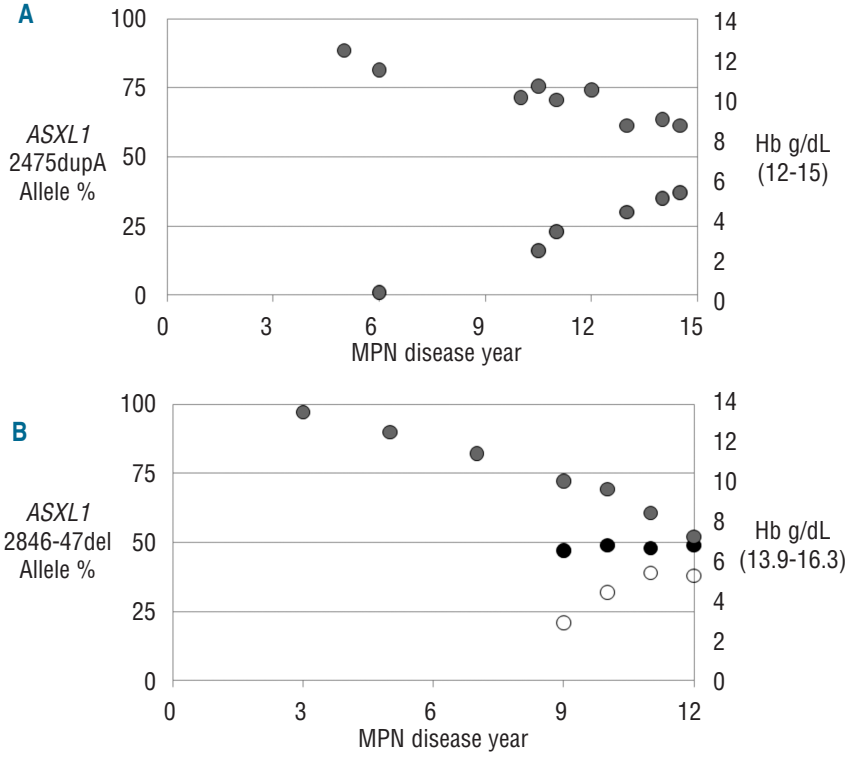

Figure 1. Sequential *ASXL1* mutant allele burdens in two MPN patients. (A) Pyrosequencing assays to detect *ASXL1* 2475dupA (gray circles) in patient UPIN 488 were negative at year 6, but subsequently were positive at increasing percentages in samples available from years 10-14, with a maximum value of 37%. The patient's clinical phenotype evolved over a 14-year course from *JAK2* V617F-negative ET, followed by a phase of myelofibrosis, terminating in AML at year 14. Her disease was marked by progressive anemia [hemoglobin concentration (Hb) in red circles] and thrombocytopenia. Treatments during her MPN phase included hydroxyurea and anegrelide. (B) Pyrosequencing assays to detect both *JAK2* V617F (black circles) and *ASXL1* 2846-47del (white circles) were both positive at years 9-12 in patient UPIN 453. *JAK2* V617F allele % was between 47-53% at all time points, while the *ASXL1* 2846-47del increased from 21 to 38% between years 9 and 12. The patient's clinical phenotype evolved over a 12-year course from ET, followed by myelofibrosis at year 9, with progressive anemia [hemoglobin (Hb) in red circles] and thrombocytopenia. The patient did not receive treatments with the exception of red cell transfusion (year 12), during this period.

leukemic blasts were not available from any of the *ASXL1*-lesion positive patients for analysis.

Serial analysis of ASXL1 *mutation-positive cases*

To test the relationship of *ASXL1* lesions with the natural history of MPN, we determined whether mutations evident in granulocytes were consistently present in samples obtained at varying time points and, when possible, measured the percentage of *ASXL1*-mutant alleles using pyrosequencing-based quantitative assays. In two patients with *ASXL1*-mutation positive post-ET-MF we demonstrated the relationship of the *ASXL1*-mutant allele burden over time and its relationship to disease features and where relevant, *JAK2* V617F allele burden (Figure 1). These studies indicated that *ASXL1* lesions are associated with the transition of ET to myelofibrosis, and increase over time independently of the *JAK2* V617F allele burden (Figure 1). The most common *ASXL1* nonsense lesion, *ASXL1* 1934dupG, cannot be assessed using pyrosequencing assays because of its repetitive nucleotide sequence preceding the 1934 insertion. However, electropherograms generated from serial samples from a single patient indicate that *ASXL1* 1934dupG also increases in intensity over time, analogous to other *ASXL1* nonsense lesions (*Online Supplementary Figure S2*). Moreover, in a single patient with MPN who harbored two distinct *ASXL1* nonsense lesions (UPIN 1327) in his granulocytes during the MPN phase, including the 1934dupG, neither *ASXL1* lesion was detected in his buccal DNA obtained after bone marrow transplantation and sustained donor engraftment (*Online Supplementary Figure S3*). These data indicate that *ASXL1* lesions accumulate over time in patients whose disease evolves from ET to post-ET-MF, associate with the myelofibrotic phase of MPN and, once present, do not appear to extinguish but rather gain in clonal representation as assessed qualitatively by direct sequencing and quantitatively by pyrosequencing. Moreover, *ASXL1* nonsense lesions 2475dupA, 2846_47del and 1934dupG are acquired, somatic lesions.

Discussion

We found that disruption of the *ASXL1* gene is prevalent in myelofibrosis due to large 20q11 deletions that included the *ASXL1* gene or due to acquired somatic mutation of *ASXL1*. *ASXL1* lesions, either deletions or nonsense mutations, were present in 36% of cases of myelofibrosis, but were identified only rarely in cases of ET or PV. In two cases, we demonstrated that *ASXL1* mutations were clearly acquired, and appeared to accumulate in intensity over time as these patients progressed from having ET to post-ET-MF. Since the residual allele was intact in the five patients in whom *ASXL1* was contained within the 20q deletion region, and the majority of *ASXL1* lesions were nonsense lesions, it appears that *ASXL1* haploinsufficiency may contribute to disease pathogenesis in PMF, post-ET-MF and post-PV-MF. With respect to clinical phenotype, there was a higher prevalence of anemia-directed therapy in *ASXL1* lesion-positive patients with myelofibrosis than in those with *ASXL1* wild-type. *ASXL1* lesions were, however, found in cases without exposure to chemotherapy (hydroxyurea), indicating that these lesions are an acquired genetic event that is related to intrinsic genomic instability, and not a specific consequence of therapy-induced genomic damage.

Our results complement previously published findings by Carbuccia *et al.*[21] who identified *ASXL1* mutations in three of ten PMF cases and one case of AML arising out of post-ET-MF, and in only one of 45 cases of ET and PV. Though prevalent in our series of patients with myelofibrosis, disruption of *ASXL1* has also been identified in other myeloid neoplasms. *ASXL1* mutations were found in 43% (19/44) of patients with chronic myelomonocytic leukemia patients (CMML) and in a higher subset of patients with myeloproliferative-CMML (13/21; 62%) compared to in patients with myelodysplastic-CMML (4/18, 22%) and acutely transformed CMML (2/7, 22%).[7] More recently, *ASXL1* mutations were found in 6/41 cases of chronic myeloid leukemia, either in chronic phase or blast crisis (in one patient with paired samples it was found in the blast crisis sample and not in the chronic phase sample), further substantiating a claim that this mutation could play a role in the pathogenesis of MPN.[9] *ASXL1* lesions have been reported in other aggressive myeloid neoplasms including high-risk myelodysplastic syndromes (17/35 with refractory anemia with excess blasts-1 or 2; 48%),[10] more recently in a large survey of cases of myelodysplasia (40/193; 20.7%),[22] and in AML (54/501; 11%).[6] The specific role of *ASXL1* haploinsufficiency in the pathophysiology of acute or chronic myeloid neoplasms is not currently understood. Targeted disruption of the *ASXL1* gene generating a haploinsufficient state in mice did not lead to a myelodysplastic syndrome, MPN or AML phenotypes.[23] Thus, either the gene plays a much more important role in human myeloid disease than in mice, or the haploinsufficient state functions as a cooperative lesion with other lesions, with individual phenotypes occurring as a result of the combination of many unique lesions.

ASXL1 lesions may be associated with increased genetic instability; either directly due to loss of function in their role as an epigenetic regulator, or indirectly through their association with other myeloid lesions or other factors, and in either case may be associated with specific myeloid sub-phenotypes within disease classes. For example, within AML the majority of *ASXL1* mutation-positive cases occurred with other *bona fide* high-grade AML-specific mutations or chromosomal rearrangements (39/54, 72%) and were associated with a shorter survival. However, a multivariate analysis did not show that *ASXL1* status was an independent prognostic factor.[6] To the contrary, in a recent large series of patients with myelodysplastic syndromes, *ASXL1* frameshift lesions were independently associated with reduced survival and a shortened time to evolution into AML.[22] With regard to an association between *ASXL1* lesions and AML development in the current MPN series, *ASXL1* lesions were no more prevalent in the patients who subsequently developed AML than in the myelofibrosis patients as a whole, suggesting that *ASXL1* does not directly cause a leukemic phenotype. *ASXL1* lesions in both the AML cohort and this MPN cohort were associated with older age and male sex (Table 1).

It has been suggested that the most commonly reported mutation within *ASXL1*, the 1934dupG frameshift lesion, is not a somatic alteration given its detection in paired tumor and buccal smears from MPN patients, and its reported identification in individuals without myeloid disease.[24] In our study and in the AML study, *ASXL1* 1934dupG was detected in disease phases but not remis-

sion phases in evaluable patients and we specifically show its acquisition and increasing intensity over time in sequential studies (*Online Supplementary Figures S1 and S2*); these data are not compatible with *ASXL1* 1934dupG as a constitutional allele. The use of buccal smears as a source of control tissue is problematic, as we have easily detected *JAK2* V617F in buccal preparations from *JAK2* V617F-positive MPN patients, which may be a result of saliva or plasma contamination (*data not shown*). Furthermore, if *ASXL1* 1934dupG was merely a germline alteration, we would have expected a higher prevalence in our ET and PV patients, whereas only 1 of 83 patients was found to have *ASXL1* 1934dupG. Moreover, the *ASXL1* 1934dupG frameshift is not a common sequence variation in either the dbSNP or in the 1000 Genomes Project, nor was it identified in 65 healthy volunteers sequenced for *ASXL1* by Thol *et al.*[22] Additionally, Thol *et al.* cloned the mutated region of *ASXL1* and identified the mutant and the wild-type clone in all nine patients with c.1934dupG.[22] These observations are inconsistent with the behavior of an artifact or a germline mutation, and challenge the assertion that the 1934dupG is not an acquired somatic alteration.

In the context of the classical *BCR-ABL*-negative MPN, *ASXL1* mutation or deletion implies a unique association with myelofibrosis, an entity with a historically significantly shorter survival than that of ET or PV. We identified an association with a need for anemia-directed therapy (potentially associating with more severe anemia) in *ASXL1*-mutated patients, which often suggests a worse prognosis, given the significance of anemia to leukemic transformation and overall survival in myelofibrosis.[2,25-27] However, the fact that in our series patients with *ASXL1* lesions survived longer than the median disease durations argues against an association with a poor prognosis. Furthermore, *ASXL1* lesions did not appear to accelerate transformation from ET or PV to myelofibrosis, did not accelerate transformations of myelofibrosis to AML, and were not overrepresented in AML cases. In fact, deletions of 20q have been historically classified as "favorable."[28,29] Given the wide range of natural history and survival even within cases of myelofibrosis,[30] prospective studies with longer follow-up will be required in order to clarify any differences in prognosis between *ASXL1* mutant and wild-type cases.

In conclusion, *ASXL1* lesions are prevalent in myelofibrosis and the association of these lesions, due to either mutation or deletion, suggests that *ASXL1* haploinsufficiency is associated with a myelofibrosis phenotype in the context of other known and unknown lesions, and that disruption of *ASXL1* function may contribute directly to the pathophysiology and clinical complications of primary and secondary myelofibrosis. These data support the concepts that cooperative lesions in addition to *JAK2* V617F are critical in generating myelofibrosis, that myelofibrosis is molecularly more complex than either PV or ET, and that the transition of PV or ET to myelofibrosis is associated with the acquisition of genomic lesions that are present in myelofibrosis at large. Future studies testing the role of *ASXL1* in murine models in combination with other myeloid lesions will be useful in determining its specific relationship to the pathophysiology of these disorders. The identification of *ASXL1* as a prevalent, myelofibrosis-specific disease allele within the MPN opens new pathways for diagnostic testing, disease monitoring (particularly for the transition from ET to myelofibrosis) and therapeutic targeting of epigenetic dysregulation.

Authorship and Disclosures

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