Acquired *ASXL1* mutations are common in patients with inherited *GATA2* mutations and correlate with myeloid transformation

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ABSTRACT

Inherited or sporadic heterozygous mutations in the transcription factor *GATA2* lead to a clinical syndrome characterized by non-tuberculous mycobacterial and other opportunistic infections, a severe deficiency in monocytes, B cells and natural killer cells, and progression from a hypocellular myelodysplastic syndrome to myeloid leukemias. To identify acquired somatic mutations associated with myeloid transformation in patients with *GATA2* mutations, we sequenced the region of the *ASXL1* gene previously associated with transformation from myelodysplasia to myeloid leukemia. Somatic, heterozygous *ASXL1* mutations were identified in 14/48 (29%) of patients with GATA2 deficiency, including four out of five patients who developed a proliferative chronic myelomonocytic leukemia. Although patients with *GATA2* mutations had a similarly high incidence of myeloid transformation when compared to previously described patients with *ASXL1* mutations, GATA2 deficiency patients with acquired *ASXL1* mutation were considerably younger, almost exclusively female, and had a high incidence of transformation to a proliferative chronic myelomonocytic leukemia. These patients may benefit from allogeneic hematopoietic stem cell transplantation before the development of acute myeloid leukemia or chronic myelomonocytic leukemia. (*ClinicalTrials.gov identifier NCT00018044, NCT00404560, NCT00001467, NCT00923364.*)

Introduction

Recently, four groups described a new human disease syndrome, termed GATA2 deficiency, resulting from heterozygous germline or sporadic mutations in the transcription factor *GATA2*.[1-4] Each group approached this syndrome from a distinct clinical perspective resulting in four different names for the same syndrome: autosomal dominant and sporadic monocytopenia and mycobacterial infection (MonoMAC);[3,5] dendritic cell, monocyte, B and NK lymphoid (DCML) deficiency;[1] Emberger syndrome with lymphedema and monosomy 7;[4] and familial myelodysplastic syndrome (MDS)/acute myelogenous leukemia (AML).[2] The constellation of disease manifestations for GATA2 deficiency includes: (i) human papillomavirus, non-tuberculous mycobacterial and other opportunistic infections; (ii) severe deficiency of monocytes, dendritic cells, B cells, and NK cells in the peripheral blood, (iii) sporadic or autosomal dominant inheritance; (iv) the propensity to transform from a hypocellular MDS to AML or chronic myelomonocytic leukemia (CMML).

The late and variable tendency for GATA2 deficiency patients to develop AML/CMML invokes the Knudsen hypothesis of multiple *de novo* mutations driving progression to cancer.[6,7] However, it has not been determined whether the genetic lesions associated with the pathogenesis of *de novo* AML are the same as those driving leukemic transformation when the initiating "hit" is discrete, as in *GATA2*-associated AML. Mutations in *ASXL1* (*Additional sex combs-like 1* gene) have been associated with the transformation of MDS to AML, and particularly, CMML.[8-11] Transformation from MDS to AML/CMML is also common among GATA2 deficiency patients. Furthermore, *ASXL1* mutations were reported in two cousins with *GATA2* mutations.[12] We, therefore, investigated whether *ASXL1* mutations were a common "second hit" among GATA2 deficiency patients, and whether this correlated with leukemic transformation. *ASXL1*, the mammalian homolog of the *Drosophila additional sex combs* gene, is an essential component of two distinct chromatin-modifying complexes and is expressed in hematopoietic cell lineages.[13]

We identified mutations in *ASXL1* in 14/48 (29%) of GATA2 deficiency patients, including four patients who progressed to CMML. We found notable differences in the clinical and biometric features in GATA2 deficiency patients with MDS/AML and patients with MDS/AML without preceding *GATA2* mutations.

Methods

ASXL1 exons 12 and 13 (NCBI: NM_015338) were analyzed by direct sequencing in 48 patients with *GATA2* mutations. A polymerase chain reaction (PCR) was used to amplify a 4,287 bp segment of *ASXL1* with several overlapping primer sets (*Online Supplementary Table S1*). Substrate DNA was isolated from mononuclear and granulocyte cell preparations from peripheral blood or bone marrow aspirates using Gentra Puregene or DNeasy Blood & Tissue Kits (QIAGEN Sciences, Germantown, MD, USA), or from extracts prepared from microscope slides of unfixed, unstained bone marrow aspirates using the Epicentre BuccalAmp DNA Extraction Kit (Madison, WI, USA)

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(*Online Supplementary Table S2*). The PCR were done with AccuPrime *Taq* DNA Polymerase High Fidelity (Life Technologies, Grand Island, NY, USA) using the manufacturer's recommended conditions with 30 ng of substrate DNA and 35 amplification cycles, with the following amplification parameters: 94°C for 20 seconds; 58°C for 30 seconds, and 68°C for 60 seconds/kilobase of amplified product. The p.G646Wfs*12insG mutation was verified first by repeating the PCR using two different primer sets (*Online Supplementary Table S1*: primers 1/4, primers 2/3), then by repeating the reactions with different polymerase enzymes [AccuPrime *Pfx* (Life Technologies, Grand Island, NY, USA), *DreamTaq* (Thermo Scientific, Pittsburgh, PA, USA)] using the manufacturers' recommended conditions. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN Sciences, Germantown, MD, USA) and sequenced using ABI 3130XL and 3730 fluorescence-based sequencers. Sequences were analyzed with MacVector, version 12.0 (MacVector, Inc, Cary, NC, USA). Mutations were confirmed with independent PCR with separate primer sets. Statistical analyses were done with GraphPad Prism, version 5.0 (GraphPad Software, Inc., La Jolla CA, USA). The clinical protocols under which these studies were undertaken were reviewed and approved by the Institutional Review Board of the National Institutes of Health, Clinical Research Center. Patients in this study were enrolled in National Institutes of Health/National Institute of Allergy and Infectious Diseases ClinicalTrials.gov Identifier: NCT00018044, NCT00404560, NCT00001467, and National Cancer Institute ClinicalTrials.gov Identifier: NCT00923364.

Results and Discussion

We screened 48 patients with inherited *GATA2* mutations to determine the incidence of somatic *ASXL1* mutations. A schematic of the *ASXL1* region that was sequenced, which contains ~90% of known *ASXL1* mutations (COSMIC, v66[14]), and the position of the mutations found in this study, are shown in Figure 1A. Sequence variations found in the Database of Single Nucleotide Polymorphisms (dbSNP) were not included as mutations

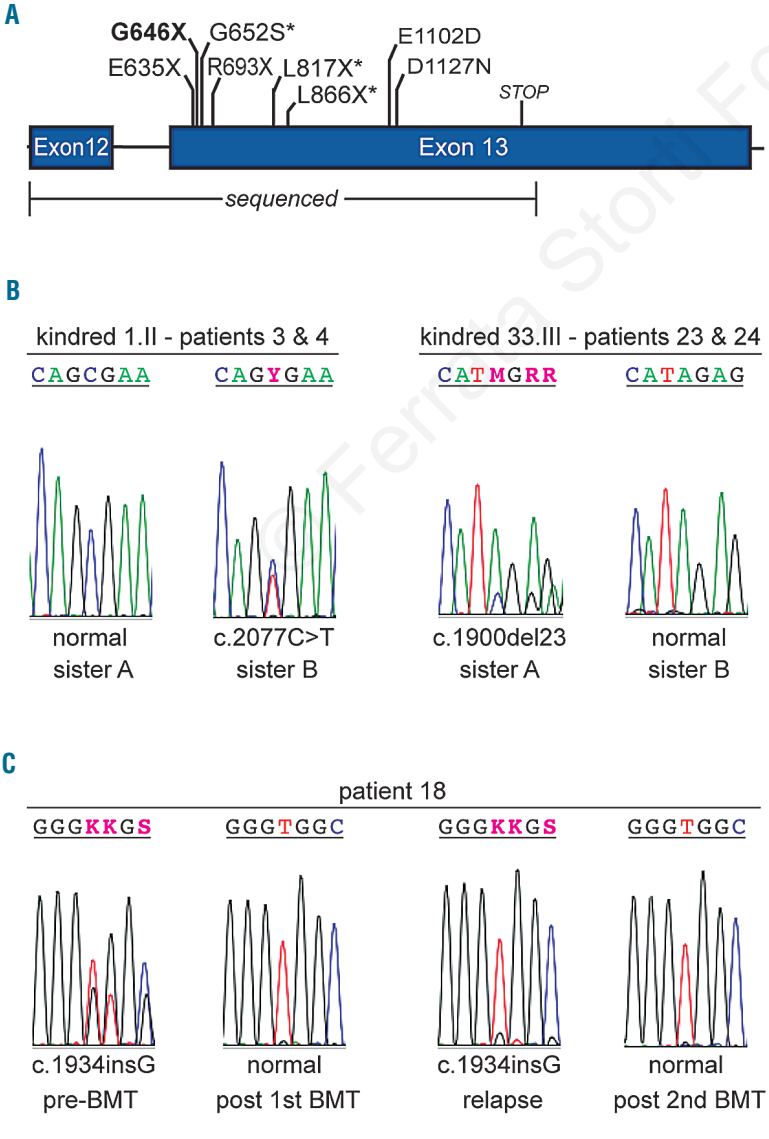

Figure 1. ***ASXL1*** **mutations in a cohort of GATA2 deficiency patients. (A) Diagram of the 3'-terminal two exons of the *ASXL1* gene (NC_000020 REGION: 30946147.31027122) indicating the regions sequenced and the mutations found in this cohort of patients. An (*) indicates a novel mutation identified in this study. (B) Discordance in *ASXL1* mutations between two sets of sisters; each sister had the identical *GATA2* mutation. In kindred 1.II, the patients had different *ASXL1* genotypes, with sister A displaying a normal sequence at the locus where sister B had a c.1192C>T mutation. Kindred 33.III shows sister A (patient 23) with an *ASXL1* c.1900del23 mutation while sister B (patient 24) has no *ASXL1* mutation. The International Union of Pure and Applied Chemistry (IUPAC) notation is used to designate nucleotide sequence. (C) Example of disease progression and *ASXL1* mutation profile in patient 18. Sequence traces show patient 18 with *ASXL1* c.1934insG mutation prior to bone marrow transplantation. This mutation was not detectable after a non-myeloablative transplant from her normal sibling, re-appeared at relapse, and became undetectable again after a second, myeloablative transplant from the same donor. Kindred designations are listed in *Online Supplementary Table S2*.**

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(*Online Supplementary Table S3*). Somatic *ASXL1* mutations were detected in 14/48 (29%) patients with GATA2 deficiency (Table 1). All of these mutations were heterozygous and located within exon 13. The *ASXL1* mutations found among GATA2 deficiency patients were similar to mutations previously reported in MDS and AML patients,[15] including five independent cases of the most frequently described *ASXL1* mutation (p.G646Wfs*12insG). The p.G646Wfs*12insG mutation was previously reported in two cousins with a *GATA2* mutation who had developed MDS.[12] However, there has been concern over the validity of this particular mutation, with suggestions that it is a PCR artifact since it occurs immediately 3' to an eight base poly G sequence.[16] We confirmed this mutation by

Table 1. Clinical diagnosis and *GATA2/ASXL1* mutation status of patients with GATA2 deficiency.^

#	Sex	Age	*GATA2* mutation	Cytogenetics	Disease	*ASXL1* mutation
1	M	33	p.T354M - c.1061C>T	trisomy 8	MDS	none
2	M	36	c.1017+572C>T	normal	MDS	none
3[A]	F	49†	p.R398W – c.1192C>T	ND	CMML	p.G646fs – c.1934insG
4[A]	F	46†	p.R398W – c.1192C>T	normal	CMML	p.R693X – c.2077C>T
5[B]	F	53†	c.1017+572C>T	normal	MDS	none
6[B]	M	78	c.1017+572C>T	normal	CMML	none
7	M	33	p.G82fs – c.243_244delAinsGC	normal	MDS	none
8	F	59†	p.R398W – c.1192C>T	normal	CMML	p.G646fs – c.1934insG
9	F	42†	p.T354M - c.1061C>T	dicentric 6 trisomy 8	MSD/AML	none
10	M	28†	p.R361del4 – c.1083_1094del12	monosomy 7 trisomy 8	MDS	p.G652S – c.1954G>A *
11[C]	M	34†	c.1-200_871+527del2033ins7	monosomy 7	MDS	none
12[C]	F	60	c.1-200_871+527del2033ins7	normal	Emberger	none
13	F	31	c.1017+572C>T	normal	MDS	p.D1127N – c.3379G>A
14	F	38	p.N371K – c.1113C>G	monosomy 7	MDS	p.L817fs – c.2448delT *
15	M	15	p.D259fs – c.769_778dup	monosomy 7	MDS	none
16	F	23	p.R396Q – c.1187G>A	trisomy 8	MDS	none
17	M	33	p.T354M - c.1061C>T	trisomy 8	MDS	none
18	F	47	del340-381 c.1018-1G>A	t(1;22)	MDS	p.G646fs – c.1934insG
19	F	22†	p.G101fs – c.302delG	monosomy 7 trisomy 8	CMML	p.G646fs – c.1934insG
20[C]	M	15	p.C373del5 – c.1116_1130del15)	monosomy 7	MDS	none
21[C]	F	50	p.C373del5 – c.1116_1130del15)	normal	MDS	none
22	M	55	p.R396Q – c.1187G>A	normal	low B cell	none
23[A]	F	17	p.D367fs – c.1099inG	normal	MDS	p.E635fs – c.1900_1922del23
24[A]	F	10	p.D367fs – c.1099inG	ND	monocytopenia	none
25	F	35	c.1017+512del28	normal	MDS	none
26[D]	M	32	p.R396Q – c.1187G>A	normal	MDS	none
27[D]	M	29	p.R396Q – c.1187G>A	normal	MDS	none
28	F	46	p.G199fs – c.586_593dup	normal	MDS	p.G646fs – c.1934insG
29	F	48	5'UTR	trisomy 8	MDS	none
30[E]	F	25	p.R330X – c.988C>T	trisomy 8	MDS	none
31[E]	F	25	p.R330X – c.988C>T	trisomy 8	MDS	none
32	F	13	p.R396W – c.1186C>T	trisomy 8	MDS	none
33	F	24	p.V140fs – c.417dupT	normal	MDS	p.E635fs – c.1900_1922del23
34	M	15	p.R330X – c.988C>T	monosomy 7	MDS	none
35	M	22	p.R398W – c.1192C>T	normal	MDS	none
36	F	24	c.1017+512C>T	normal	MDS	p.L866X – c.2597T>G *
37	F	33	p.R361C - c.1081C>T	normal	MDS	none
38	M	--	p.L375F – c.1123C>T	unknown	unknown	none
39	F	25†	p.A318fs – c.941_951dup	monosomy 6	MDS	p.E1102D–c.1900_1922del23
40[B]	F	43	p.M388T – c.1163T>C	normal	monocytopenia	none
41[B]	M	77	p.M388T – c.1163T>C	normal	laryngeal cancer	none
42	F	44	p.R337X – c.1099C>T	monosomy 7	MDS/Emberger	p.E635fs – c.1900_1922del23
43	M	24	p.R396W – c.1186C>T	normal	MDS	none
44	M	26	p.F265fs – c.793_802del10	normal	MDS	none
45	F	64†	unknown	trisomy 8	MDS	none
46	F	12	p.R396Q – c.1187G>A	normal	MDS	none
47	M	33	p.R398W – c.1192C>T	delete Y	MDS	none
48	M	31	pR361H – c.1082G>A	normal	MDS	none

^Patients listed in the chronological order of analysis; †Deceased; [A]sisters; [B]daughter/father; [C]son/mother; [D]brothers; [E]monozygotic twins; *mutation unique to this study. ND: not determined.

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repeating the PCR at least three times for each positive sample (for patient 18, more than 12 times) with different primer pairs, and using different enzymes for amplification in the PCR (see *Methods* section). In each case, the mutation was consistently present in the positive samples, and was not observed in normal controls. Moreover, the presence of the mutation correlated with the clinical course in two patients (Figure 1C, *Online Supplementary Figure S1*). There were also three cases of the second most common *ASXL1* mutation (p.E635fs*15del23) (COSMIC, v66[14]). These two mutations have been reported at similar rates in previous studies of AML and CMML.[10,11,17-21] The remaining six mutations were found once each. Three of them were previously unreported: G652S, L817fs*1, and L866X. There was no correlation between the presence or type of somatic *ASXL1* mutation and the specific germline *GATA2* mutation; the eight different *ASXL1* mutations were found in ten different *GATA2* mutant backgrounds (Table 1).

Two pairs of sisters with the same *GATA2* mutations had discordant *ASXL1* genotypes (Figure 1B, Table 1). The first pair of sisters (kindred 1.II, patients 3 and 4) both progressed to CMML, but one had the somatic *ASXL1* c.2077C>T mutation, and the other a possible c.1934insG mutation. In the second pair of sisters (kindred 33.III, patients 23 and 24), one had an *ASXL1* mutation and MDS (patient 23), whereas the younger sister did not have either an *ASXL1* mutation or MDS (patient 24).

The *ASXL1* mutation was also followed through disease progression and treatment in two patients (Figure 1C). The c.1934insG mutation was detected in patient 18 prior to treatment, became undetectable following non-myeloablative hematopoietic stem cell transplantation, re-emerged with relapse, and became undetectable again following a second, myeloablative transplant. This re-appearance of an *ASXL1* mutation upon relapse has also been seen in AML patients,[18,20] indicating that it is a stable mutation. Patient 19 showed an increase in the mutation signal over time, which correlated with progression from MDS to a terminal, proliferative CMML (*Online Supplementary Figure S1*).

Several biological features characterized patients with GATA2 deficiency who harbored *ASXL1* mutations (Figure 2A). *ASXL1* mutations were found in four of five patients with GATA2 deficiency who developed CMML, consistent with the previously reported high incidence of *ASXL1* mutations in CMML.[9,10,21,22] Moreover, when Gelsi-Boyer *et al.*[10,23] divided their group of patients into those with dysplastic (CMML-MD) and proliferative (CMML-MP) forms of CMML, *ASXL1* mutations were present in 76% of CMML-MP cases *versus* 27% of CMML-MD cases. The CMML patients in this report all had CMML-MP. While *ASXL1* mutations were prevalent in patients who developed CMML, the presence of an *ASXL1* mutation was not a predictor for the development of MDS: 27/37 (73%) patients who developed MDS lacked *ASXL1* mutations.

Several studies have shown that the average age of

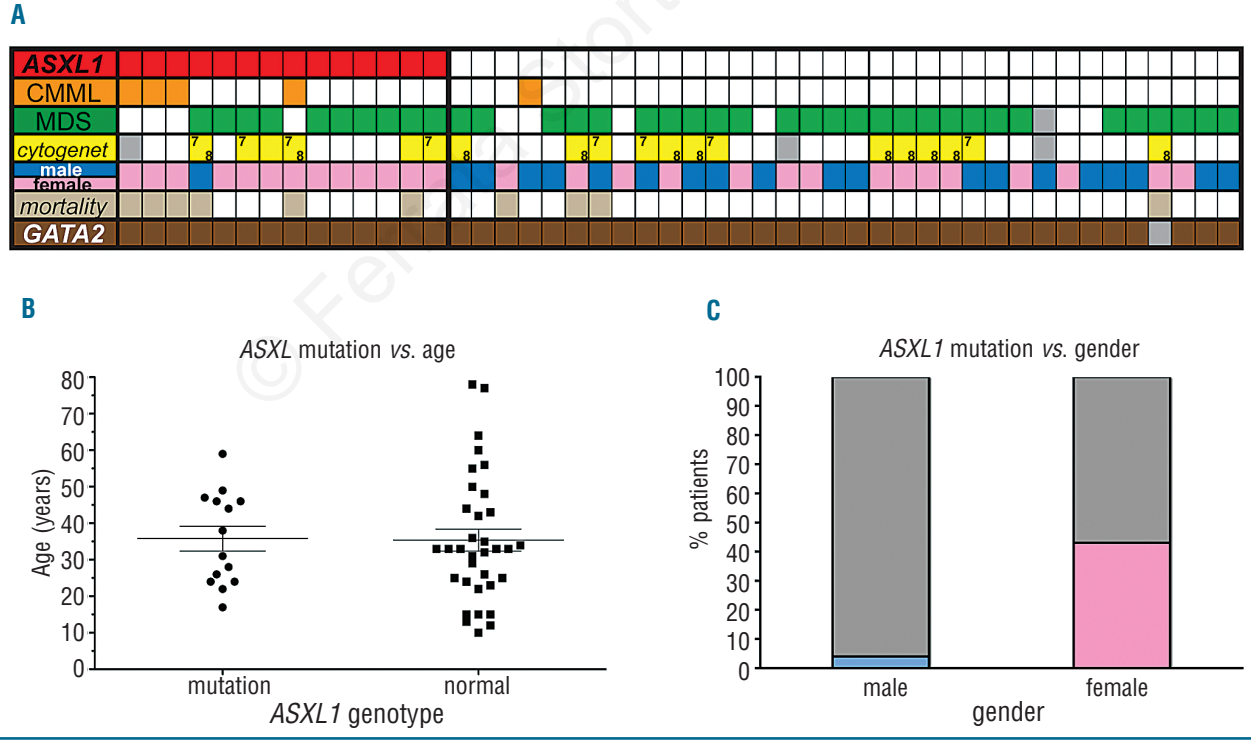

Figure 2. Biometric parameters of GATA2 deficiency patients. (A) Summary of the *ASXL1* mutation status, clinical diagnosis, and biometric characteristics of the 48 patients in this study. Patients with *ASXL1* mutations are grouped together, followed by patients without *ASXL1* mutations. Within each group, patients' samples are shown in chronological order of analysis, as listed in Table 1 and *Online Supplementary Table S2*. A yellow box indicates unfavorable cytogenetics, with "7" or "8" indicating the presence of monosomy 7 or trisomy 8, respectively. A gray box indicates information that is not available. (B) Patients' age at sample collection for patients with and without *ASXL1* mutations. Means and standard errors are shown for each data set. There is no statistical difference between the means (P=0.81) or the variance (P=0.26). (C) Percentages of male and female patients with an *ASXL1* mutation (colored boxes) and the percentages without a mutation (gray boxes) out of the total number of patients of each gender.

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MDS/AML patients with *ASXL1* mutations is higher than that of patients without an *ASXL1* mutation.[9,18-20,24,25] In general, *ASXL1* mutations are also rare in pediatric AML.[26,27] However, this was not seen in GATA2 deficiency patients. The average age of GATA2 deficiency patients with an *ASXL1* mutation (35.8 ± 12.8 years old, range, 17-59 years old) was markedly younger than that of other MDS/AML groups, but not significantly different from that of GATA2 deficiency patients without an *ASXL1* mutation (34.5±17.0 years old, range 10-78 years old) (t-test, *P*=0.81) (Figure 2B). Thus, although many gene mutations associated with MDS/AML correlate with age, the *ASXL1* mutations described here, like previously reported *FLT3*-IDT mutations,[28] are notable exceptions. Curiously, acquired *GATA2* mutations in *CEBPA*-AML patients also occur in younger patients.[29]

ASXL1 mutations in previously described MDS/AML patients show a significant bias towards male patients (~70%) when averaged over several previous studies.[11,18-21,25,30] In contrast, 13/14 (93%) of the GATA2 deficiency patients with *ASXL1* mutations were female (Figure 2C). Moreover, four of the five GATA2 patients with CMML were female and had an *ASXL1* mutation. The male CMML patient did not have an *ASXL1* mutation, and the male patient with an *ASXL1* mutation did not have CMML. Gender bias was not seen in the other biometric data analyzed here. Leukemic mutations in some chromatin modifiers show a modest clustering in female patients, but *ASXL1*, and its most frequently associated genes, *SRSF2* and *U2AF1*, are more frequent in males.[31]

The basis for the gender bias observed here in GATA2 deficiency patients is unknown. However, *GATA2* plays a role in the hormone-dependent recruitment of the androgen receptor to chromatin, and in this way, *GATA2* influences the expression of androgen-dependent genes, while not being directly regulated by androgen.[32] *GATA2* is also over-expressed in aggressive, metastatic prostate cancers, and its expression affects the hormone-responsive growth of these cells.[33] The significance of this in hematopoiesis and leukemia is not known, but the androgen receptor is expressed widely in the bone marrow in both males and females.[34]

Among MDS/AML patients, *ASXL1* mutations are most commonly found in patients with International Prognostic Scoring System low/intermediate 1 risk or normal cytogenetics.[11,15,19,24,26,35] Cytogenetics from 13 of the GATA2 deficiency patients with *ASXL1*-mutations showed a 46% incidence of unfavorable cytogenetics: monosomy 7 in two, monosomy 7 and trisomy 8 in two, one translocation t(1;22), and one chromosome 6 monosomy (Table 1, Figure 2A). The two GATA2 deficiency patients with *ASXL1* mutations described by Bödör *et al.* also had monosomy 7.[12] However, over 40% of the GATA2 deficiency patients (13/32) without *ASXL1* mutations had monosomy 7 (4/32) or trisomy 8 (9/32). Thus, an abnormal karyotype was not more common among patients with *ASXL1* mutations than those without the mutation. The high frequency of monosomy 7 and/or trisomy 8 among GATA2 deficiency patients makes it difficult to assess the relationship between *ASXL1* mutation and these abnormal karyotypes.

Several studies have suggested that the presence of an *ASXL1* mutation in MDS/AML patients is a predictor of rapid disease progression and poor overall survival.[8,11,15,22,25,36,37] Among the GATA2 deficiency patients, 6/14 (43%) with an *ASXL1* mutation did not survive the time period of this study. In contrast, the mortality rate was 4/34 (12%) for those without an *ASXL1* mutation. Thus, our results are consistent with earlier reports that an *ASXL1* mutations correlate with poor survival.

Mutations in the *ASXL1* gene have become a common observation in hematopoietic malignancies, particularly in sporadic MDS and AML/CMML. The mutations that drive leukemogenesis generally fall into discrete categories of function, including transcription factors, epigenetic modifiers, splicing factors, and signal transduction pathways.[7,38,39] Patients with *GATA2* mutations usually have inherited or acquired a mutation in the first category of mutation, the GATA2 transcription factor. The *ASXL1* mutation represents the second class of mutation, an epigenetic or chromatin modifier. Presumably, the patients who progress to AML and CMML have additional somatic mutations in the malignant myeloid clone.

This study indicates that mutations in *ASXL1* in patients with mutations in *GATA2* represent an important "second hit" in myeloid transformation, particularly to CMML. In this cohort of patients, mutations in *ASXL1* indicate the need for close clinical follow-up and, potentially, allogeneic hematopoietic stem cell transplantation. Recently, we have initiated whole exome analysis on GATA2 deficiency patients to further define the pattern of genetic changes that influence the tempo and phenotype of myeloid transformation.

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Authorship and Disclosures

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