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ORIGINAL REPORT

Mutations of E3 Ubiquitin Ligase *Cbl* Family Members Constitute a Novel Common Pathogenic Lesion in Myeloid Malignancies

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The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

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ABSTRACT

Purpose
Acquired somatic uniparental disomy (UPD) is commonly observed in myelodysplastic syndromes (MDS), myelodysplastic/myeloproliferative neoplasms (MDS/MPN), or secondary acute myelogenous leukemia (sAML) and may point toward genes harboring mutations. Recurrent UPD11q led to identification of homozygous mutations in *c-Cbl*, an E3 ubiquitin ligase involved in attenuation of proliferative signals transduced by activated receptor tyrosine kinases. We examined the role and frequency of *Cbl* gene family mutations in MPN and related conditions.

Methods
We applied high-density SNP-A karyotyping to identify loss of heterozygosity of 11q in 442 patients with MDS, MDS/MPN, MPN, sAML evolved from these conditions, and primary AML. We sequenced *c-Cbl*, *Cbl-b*, and *Cbl-c* in patients with or without corresponding UPD or deletions and correlated mutational status with clinical features and outcomes.

Results
We identified *c-Cbl* mutations in 5% and 9% of patients with chronic myelomonocytic leukemia (CMML) and sAML, and also in CML blast crisis and juvenile myelomonocytic leukemia (JMML). Most mutations were homozygous and affected *c-Cbl*; mutations in *Cbl-b* were also found in patients with similar clinical features. Patients with *Cbl* family mutations showed poor prognosis, with a median survival of 5 months. Pathomorphologic features included monocytosis, monocytoid blasts, aberrant expression of phosphoSTAT5, and c-kit overexpression. Serial studies showed acquisition of *c-Cbl* mutations during malignant evolution.

Conclusion
Mutations in the *Cbl* family RING finger domain or linker sequence constitute important pathogenic lesions associated with not only preleukemic CMML, JMML, and other MPN, but also progression to AML, suggesting that impairment of degradation of activated tyrosine kinases constitutes an important cancer mechanism.

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INTRODUCTION

Mutations and genomic aberrations constitute key pathogenic lesions in myeloid malignancies. In primary acute myelogenous leukemia (pAML) and chronic myelogenous leukemia (CML), reciprocal translocations have enhanced our understanding of molecular pathogenesis, improved diagnosis and provided rational therapeutic targets. In myelodysplastic syndromes (MDS), myelodysplastic/myeloproliferative neoplasms (MDS/MPN), and AML evolved from MDS or MDS/MPN (secondary AML [sAML]), unbalanced chromosomal lesions predominate and loss of heterozygosity (LOH) is of particular importance. LOH can arise either via hemizygous deletion, where a DNA segment is lost from one homolog while the other remains at one copy per cell, or by uniparental disomy (UPD), wherein the retained homolog is duplicated to preserve two total copies per cell at the locus. Thus, analysis of recurrent regions of LOH may point toward the presence of important mutations. Mutations seen in MDS and AML affect specific classes of genes and indicate general pathways of leukemia evolution.[1] For example, mutations have been found in a variety of receptor tyrosine kinases, including *c-Kit*, *c-Mpl*, and *Flt-3*.[2-4] Mutations also affect signal transduction genes such as *Jak2* and

NPM-1. *tP53* constitutes an example of a proapoptotic tumor suppressor gene mutated in aggressive leukemias.[5-7]

Until recently, metaphase cytogenetics was applied for detection of chromosomal defects including deletions resulting in LOH. Using this technique, a number of invariant chromosomal abnormalities have been described and minimal affected regions delineated, pointing towards potentially pathogenic genes. Single nucleotide polymorphism array (SNP-A) –based cytogenetic analysis allows for better resolution of chromosomal defects, with identification of previously cryptic unbalanced lesions.[8] In particular, SNP-A is able to identify UPD. We and others have recently shown that somatic UPD affecting various chromosomes can be found frequently in MDS, MDS/MPN, and sAML and have identified a number of recurrent areas.[9-11] Initially, UPD9p was shown to lead to homozygosity of the *Jak2* V617F mutation in MPN.[12-14] Using SNP-A, we have demonstrated that other areas of UPD can also be associated with homozygous mutations, including UPD13p (*Flt-3* ITD) and UPD1p (*c-Mpl*).[15,16] Based on this paradigm, we recently identified a novel recurrent area of UPD at 11q, frequently present in chronic myelomonocytic leukemia (CMML) and AML evolved from atypical MDS/MPN, and through delineation of a commonly deleted region have identified mutations in *c-Cbl*.[15] *c-Cbl* is an E3 ubiquitin ligase involved in degradation of activated receptor tyrosine kinases and other tyrosine kinases, including Src kinases. Consequently, mutations affecting the RING finger domain (RFD) may have a wide range of effects on proliferation regulation, crucial to both MPN and AML. In animal studies, *c-Cbl* knockout led to hyper-responsiveness to ligand stimulation and expansion of them in cell pools, overall resulting in a mild proliferative phenotype.[17] However, RFD mutation knock-in in a *c-Cbl*−/− mouse model resulted in a myeloproliferative phenotype and leukemic evolution (W.Y. Langdon, personal communication, December 2008). In a transgenic MDS NUP98/HOX13 mouse model, progression to sAML with acquisition of *RAS* and *c-Cbl* mutations occurs frequently.[18]

We hypothesized that mutations inactivating oncogene degradation pathways may constitute a new class of molecular lesions in myeloid malignancies modifying current paradigms of leukemogenesis. We therefore investigated the presence of mutations in the *Cbl* family of E3 ubiquitin ligases in selected subtypes of malignant myeloid disorders and determined the phenotypic and functional features as well as clinical outcomes. Based on the study of these features we set out to discern the pathophysiologic principles of molecular dysfunction created by E3 ubiquitin ligase lesions.

METHODS

Patients

Bone marrow aspirates were collected from 442 patients with MDS (n = 115), MDS/MPN (n = 98), MPN (n = 22), sAML (n = 110) evolved from these conditions, and pAML (n = 97) seen at Cleveland Clinic and Johns Hopkins Hospital between 2003 and 2008 (Appendix Table A1, online only). Informed consent for sample collection was obtained according to protocols approved by institutional review boards. Diagnosis was confirmed at each primary institution and assigned according to WHO classification criteria.[19]

Single Nuclotide Polymorphism Array Analysis

High-density Affymetrix SNP-A (250K and 6.0 arrays; Affymetrix, Santa Clara, CA) were applied as a karyotyping platform to identify LOH on chromosome 11q. Lesions identified by SNP-A were compared to the Database of Genomic Variants[20] (http://projects.tcag.ca/variation/) and an internal control series (n = 1,003) to exclude known copy number variations. To confirm all regions of LOH detected by 250K SNP-A, we repeated samples when possible on 6.0 arrays and analyzed using Genotyping Console version 2.0 (Affymetrix). Signal intensity was analyzed and SNP calls determined using Gene Chip Genotyping Analysis Software version 4.0 (GTYPE, Affymetrix). Copy number and areas of UPD were investigated using a Hidden Markov Model and CN Analyzer for Affymetrix GeneChip Mapping 250K arrays (CNAG version 3.0) as previously described.[21]

E_3 Ubiquitin Ligase Mutational Screening

To screen patients for mutations in *c-Cbl*, *Cbl-b*, *Cbl-c*, and *Hakai*, direct genomic sequencing of all exons was performed (details of primers and conditions are available on request). For sequencing, 250 ng of polymerase chain reaction (PCR) product, 3 μmol/L original forward or reverse primer, 2 μL Big Dye version 3.1 (Applied Biosystems, Foster City, CA), and 14.5 μL deionized H_2O were amplified under the following conditions: 95°C (2 minutes) followed by 25 cycles of 95°C (10 seconds), 50°C (5 seconds), and 60°C (4 minutes). Sequencing was performed as previously described.[15] If a mutation was intronic, RNA was extracted by TRIzol (Invitrogen, Carlsbad, CA) and reverse transcription polymerase chain reaction performed for confirmation of splice variants.

Immunohistochemical Detection of $pSTAT_5$

Staining was performed on a Benchmark XT platform (Ventana Medical Systems, Tucson, AZ), according to the manufacturer's instructions, using mouse monoclonal antiphospho-STAT5a/b (Y694/99; Advantex BioReagents LLP, Conroe, TX) at 1:500 dilution. All stains were scored without knowledge of the clinical diagnosis or mutational status. Phospho-STAT5-positive staining (nMEG pSTAT5) was defined as previously reported.[22,23] Images were obtained via digital microscopy using an Olympus BX51 microscope (Olympus America, Melville, NY) equipped with either a UPlanFl 40×/0.75 numeric aperture (NA) or a UPlanFl 100 ×/1.30 NA objective. Images were captured using a Dage-MTI Model DC330E charge-coupled device camera (Dage-MTI, Michigan City, IN) attached to the microscope with a U-TV1X-2 video adapter (Olympus America) and a 0.45× camera coupler (Diagnostic Instruments, Sterling Heights, MI).

Statistical Analysis

Overall survival was defined as the time a patient was diagnosed with a myeloid malignancy at Cleveland Clinic or Johns Hopkins to death or last known contact, and analyzed using Kaplan-Meier statistics and Cox's proportional hazards model. For comparison of the frequency of clinical features between *Cbl* family mutation and wild-type (WT), categoric variables were analyzed using Fisher's exact test.

RESULTS

Detection of UPD11q Using SNP-A and Detection of c-Cbl Mutation

Previously, we identified homozygous *c-Cbl* mutations in patients with CMML and UPD11q.[15] Using SNP-A karyotyping, we studied a large cohort of patients (n = 442) with MDS and related disorders, including JMML and CML blast crisis (CML-BC), to assess the frequency of this lesion within clinical subtypes. Three hundred one and 187 cases were examined by 250K and 6.0 arrays, respectively. Forty-six cases were analyzed using both arrays, yielding identical results. Based on the analysis of 1,003 controls, we determined the average size and location of nonclonal regions of autozygosity. All of these nonclonal regions were interstitial. For the purpose of this study, we have excluded all regions of autozygosity based on the size criteria (27 Mb) derived from controls. The remaining regions were confirmed by analysis of germ-line samples (nonclonal CD3+ lymphocytes) in 45 patients (Fig 1A). Regions of homozygosity found in both

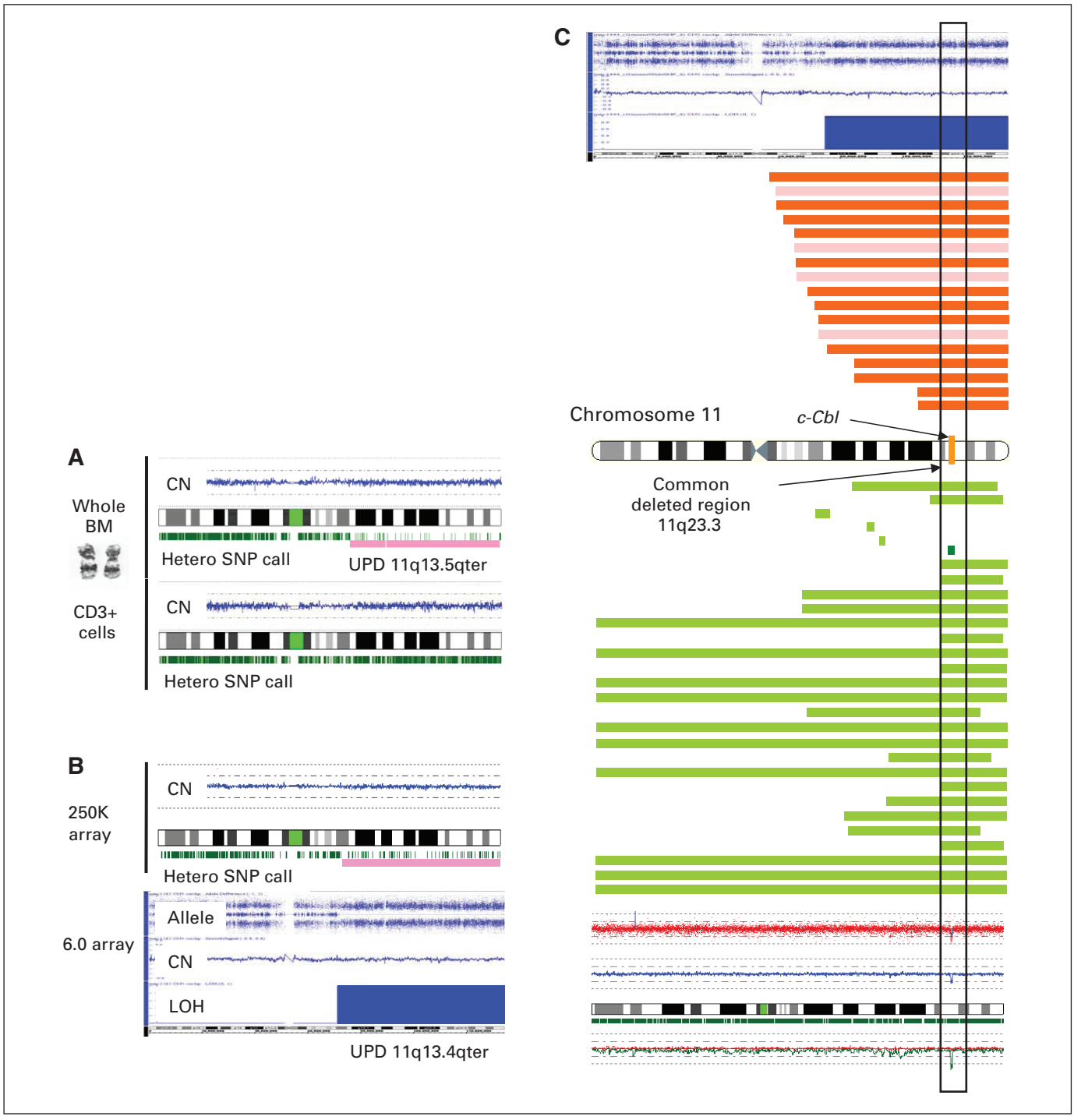

Fig 1. Detecting acquired, segmental uniparental disomy (UPD) using single nucleotide polymorphism array (SNP-A) technology. (A) SNP-A karyograms of both whole bone marrow (BM) cells and CD3+ lymphocytes of a patient show the somatic nature of acquired UPD in chromosome 11. The blue line represents average copy number (CN) signal intensity of SNPs on the array chip. In this instance, there are no CN variations, and thus, the blue line does not deviate from normal diploid CN. The green marks below the idiogram represent heterozygosity at particular DNA loci. In the region of UPD seen in whole BM cells, drastic reduction of heterozygous loci denotes the region of UPD (pink bar). The remaining green marks in the region of UPD delineate the presence of nonclonal cells in the sample. An abnormal chromosome 11 was not detected when metaphase cytogenetic analysis was performed on bone marrow. In CD3+ sorted lymphocytes, a normal chromosome 11 is seen. (B) Comparison of Affymetrix 250K and 6.0 arrays in the detection of UPD11q. CNAG version 3.0 analysis (top) shows clear UPD of chromosome 11 by loss of heterozygous loci. Repeated testing on the 6.0 array and analysis using Genotyping Console v2.0 software (bottom) confirms the 250K SNP-A findings. Note that the Genotyping Console output includes allele difference, loss of heterozygosity (LOH), and CN variation plots. The allele difference graph represents the genotypes for each individual SNP. Dots with a value of 1 represent SNPs with an AA genotype, whereas those with a value of −1 represent SNPs with a BB genotype. Dots at 0 represent heterozygous SNPs (AB). Complete loss of all AB SNPs indicates copy-neutral LOH. This is further shown by both the LOH and CN graphs, which show no loss in CN but clear LOH. (C) Topographic maps show regions of (red) UPD or (green) deletion in individual patients on chromosome 11q. Bars corresponding to the ideogram represent the regions affected for each patient. The *c-Cbl* locus was included in the common deleted region (11q23.3) among UPDs and deletions. Red and pink bars represent UPD lesions with and without *c-Cbl* mutations, while dark and light green bars show deletions with and without mutations. Top is 6.0 array karyogram with UPD11q and bottom is one with microdeletion 11q by 250K array analysis. *c-Cbl* mutations were identified in both cases.

bone marrow and CD3+ fractions were excluded from further analyses. We confirmed UPD11q detected on 250K arrays by repeated analyses using ultra-high density Affymetrix 6.0 arrays and Genotyping Console version 2.0 software (Fig 1B). Among a total of 133 regions of somatic UPD on multiple chromosomes including 1, 4, 17, and 21, UPD11q was most common (n = 17). LOH can also result from deletions, and deletions involving 11q23.3 were found in 29 patients (Fig 1C). Sequencing *c-Cbl* revealed mutations in 13 cases (76%) of UPD11q. However, among patients with deletion11q, a *c-Cbl* mutation was found in only one case (CMML). We also analyzed patients without LOH11q to assess the frequency of heterozygous mutations and identified five cases (1.2%).

c-Cbl *Mutations and Clinical Features*

We sequenced all exons of *c-Cbl*; all mutations, except for 1 frame shift mutation in the tyrosine kinase–binding domain, were associated with the RFD or linker sequence, which are highly conserved among species. More importantly, 12 mutations in the RFD were located at or next to a cysteine residue (63%; Fig 2). The presence of each somatic mutation was confirmed by bidirectional DNA sequencing of multiple isolates and comparison against CD3+ sorted lymphocytes when possible.

In one case of a patient with MDS (refractory anemia subtype) and monosomy 7 who transformed to AML, SNP-A karyotyping revealed the UPD11q, yet after transformation to AML sequencing identified a *c-Cbl* mutation creating a novel splice site resulting in a longer transcript (Appendix Fig A1, online only). We also found a hemizygous mutation in the linker sequence in a CMML patient with a microdeletion of 11q23.3 previously undetected by metaphase cytogenetics (Appendix Fig A2, online only). Eleven mutations were found in CMML and similar forms of MDS/MPN unclassifiable either at or before the time of testing; 6 of these patients progressed to sAML (55%). In total, two (5%) of 38 patients with CMML, 10 (9%) of 110 with sAML, and one (1%) of 115 patients with MDS carried mutant *c-Cbl* (Appendix Table A2, online only). In addition, *c-Cbl* mutations were found in four (19%) of 21 patients with JMML and one (10%) of 10 with CML-BC.

Other Mutations and Nonsynonimous SNPs in Related E_3 Ubiquitin Ligases

The *Cbl* family contains several other E3 ligases, including *Cbl-b* (3q) and *Cbl-c* (19q) as well as a novel member with high homology called *Hakai* (7q). Based on structural and functional similarities we hypothesized that these genes can also harbor mutations associated with similar clinical phenotypes. When we sequenced 12 patients with corresponding UPDs, no mutations were found. However, when we sequenced all patients in our cohort without *c-Cbl* mutations, we identified three with a heterozygous and one with a hemizygous *Cbl-b* mutation (Appendix Fig A3, online only) and three patients (one cell line) with a *Cbl-c* frame shift polymorphism with a single base insertion, all affecting the RFD (Fig 2). In addition, we identified 12 (7%) of 167 patients with myeloid malignancies harboring another rare nonsynonymous SNP in *Cbl-c*, also affecting the RFD (H405Y). The frequency of this SNP in the general population is less than 1% (data not shown).

Clinical Characteristics in Patients With Cbl Family Mutation

In order to identify the pathologic subtypes in which *Cbl* family member mutations may play a role, we systematically investigated a wide range of myeloid malignancies. They were most commonly associated with MDS/MPN subentities, including CMML and atypical MDS/MPN, and some cases of typical MDS. In addition, *Cbl* family mutations appear to be present in sAML with an antecedent history of MDS/MPN. Based on this distribution of *c-Cbl* mutations, we analyzed other related disease entities within MDS/MPN and MPN; we also identified *c-Cbl* mutations in JMML and in CML-BC (Appendix Table A2). This pattern suggests that *c-Cbl* mutations can be a characteristic feature of atypical MDS/MPN syndromes or serve as a second genetic hit facilitating malignant

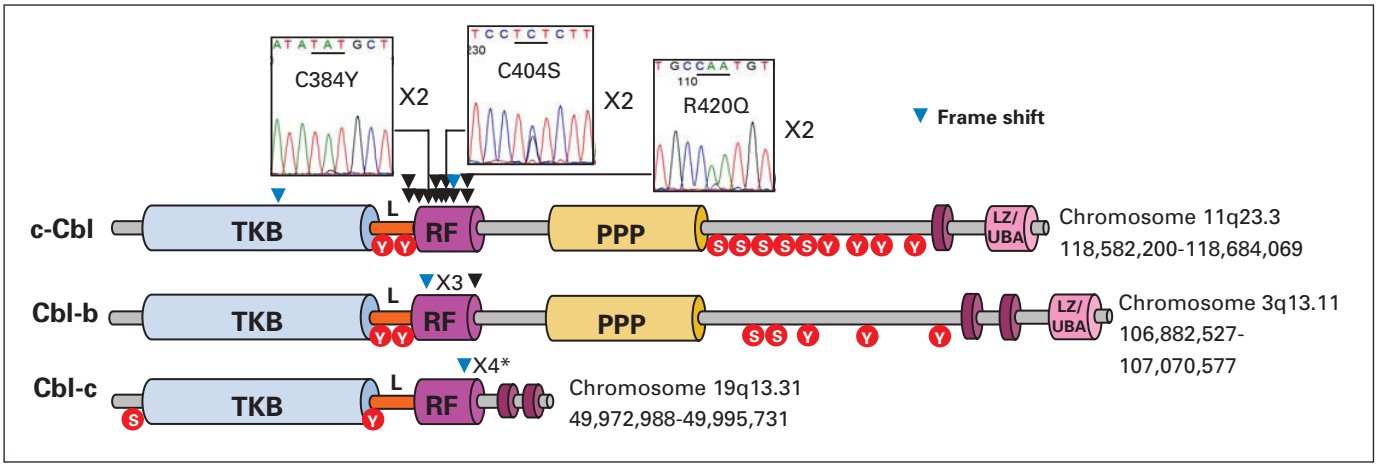

Fig 2. Identification of variations in *c-Cbl*, *Cbl-b* and *Cbl-c* RING finger (RF) domain. Schematic representation shows the major domains of *c-Cbl*, *Cbl-b*, and *Cbl-c*, primarily the tyrosine kinase binding (TKB) domain, linker sequence (L), RF domain, proline-rich region (PPP), and leucine zipper (LZ)/ubiquitin-associated domain (UBA). Tyrosine and serine residues, represented by red circles, are phosphorylated by tyrosine kinases. Genomic DNA sequencing of all exons in *c-Cbl*, *Cbl-b*, and *Cbl-c* revealed the presence of (black arrow) missense and (blue arrow) frame shift mutations or frame shift polymorphisms in L or RF domain, except for a case with a mutation in the TKB domain. In *c-Cbl*, some basepair changes occured in a homozygous state because of UPD and resulted in the substitution of cysteine or arginine residues at positions 384 shared in two patients (C384Y), 404 in three patients (C404S/Y), and 420 in three patients (R420Q/P). (*) Frame shift polymorphism.

evolution. Our study demonstrates the ubiquitous nature of *Cbl* family mutations, in particular in the context of very circumscribed phenotypes of myelomonocytic neoplasms.

Several common clinical features were identified among 27 patients affected by the *Cbl* family of variants, including monoblast or monocyte proliferation (74%), splenomegaly (81%), and surface expression of c-kit on malignant cells (93%). Characteristic nuclear features included abnormal lobulation and hyperchromatic and raisinoid nuclei of megakaryocytes seen in patients with CMML (Appendix Fig A4, online only). By immunohistochemistry, *c-Cbl* mutant megakaryocyte nuclei displayed aberrant pSTAT5 staining (86%; Appendix Fig A4), while pSTAT5 was not expressed in patients without *c-Cbl* mutations. In three cases, pSTAT5 expression was not detected in specimens obtained before the mutation was present (Appendix Fig A4). The clinical phenotype of patients with *Cbl-b* mutations was not distinguishable from that of patients with *c-Cbl* mutations. The allelic pattern of *Cbl* family mutations plays an important role in disease phenotype in patients; in sAML eight (62%) of 13 *c-Cbl* family mutations were homozygous (Fig 3A).

To evaluate the impact of *Cbl* family mutations, we first compared mutant and WT cases on the basis of various clinical parameters (Table 1). In mutant cases, monocyte counts were significantly higher as compared with patients with WT *Cbl* family genes. A higher proportion of patients with *c-Cbl* mutations were treated with intense chemotherapy or stem cell transplantation, suggesting that these therapies were more frequently selected because of the aggressive biology of the *Cbl* mutation-associated disease. When we performed univariate analysis of survival impact of various clinical variables, significant differences in WBC, monocyte counts, disease risk, and the presence of *Cbl* family gene mutations (Table 2) were found. Chemotherapy was an adverse risk factor for survival, likely as it correlated with more advanced disease in multivariate analyses. The prognosis of patients with *Cbl* family mutations was poor, especially in those with homozygous mutations. The median overall survival was 5 months for all patients, with a significant difference of 1.7 months versus 20 months for patients with homozygous and heterozygous mutations, respectively ($P = .04$; Fig 3B). In multivariate analyses advanced disease (hazard ratio [HR], 5.64; 95% CI, 3.8 to 8.36) and *Cbl* family mutations (HR, 2.17; 95% CI, 1.18 to 4.02; Table 3) were shown to be independent adverse factors for overall survival.

DISCUSSION

In leukemia, cytogenetic abnormalities identify underlying pathophysiology and carry enormous prognostic and therapeutic significance. Various examples of gain of function and inactivating mutations show that homologous recombination may lead to duplication of affected alleles.[24] Consequently, areas of somatic UPD may point toward genes carrying putative pathogenic mutations. When high density SNP-A was applied as a karyotyping platform in a large number of patients with MDS and related disorders, we noted recurrent somatic UPD at chromosome 11q, particularly frequent in MDS/MPN. Commonly deleted region mapping and analysis of genes located within this region led us to hypothesize that *c-Cbl* may contain mutations. Sequencing of *c-Cbl* in patients affected by somatic UPD11q revealed RFD and linker sequence mutations present in patients with CMML, MDS/ MPN unclassifiable, and sAML derived from these conditions or MDS. We also found new mutations in *Cbl-b* with a clinical phenotype similar to that seen with *c-Cbl* RFD mutations. Consequently, our results imply that E3 ubiquitin ligases constitute a novel class of genes in whom mutations reflect a novel general mechanism of leukemogenesis. This notion is supported by the variety of pathomorphologic subentities of myeloid malignancies affected by mutations of the *Cbl* family. Moreover, a novel frame shift polymorphism was found in *Cbl-c* in patients with MDS/MPN. However, the relevance of this otherwise extremely rare polymorphism is not clear

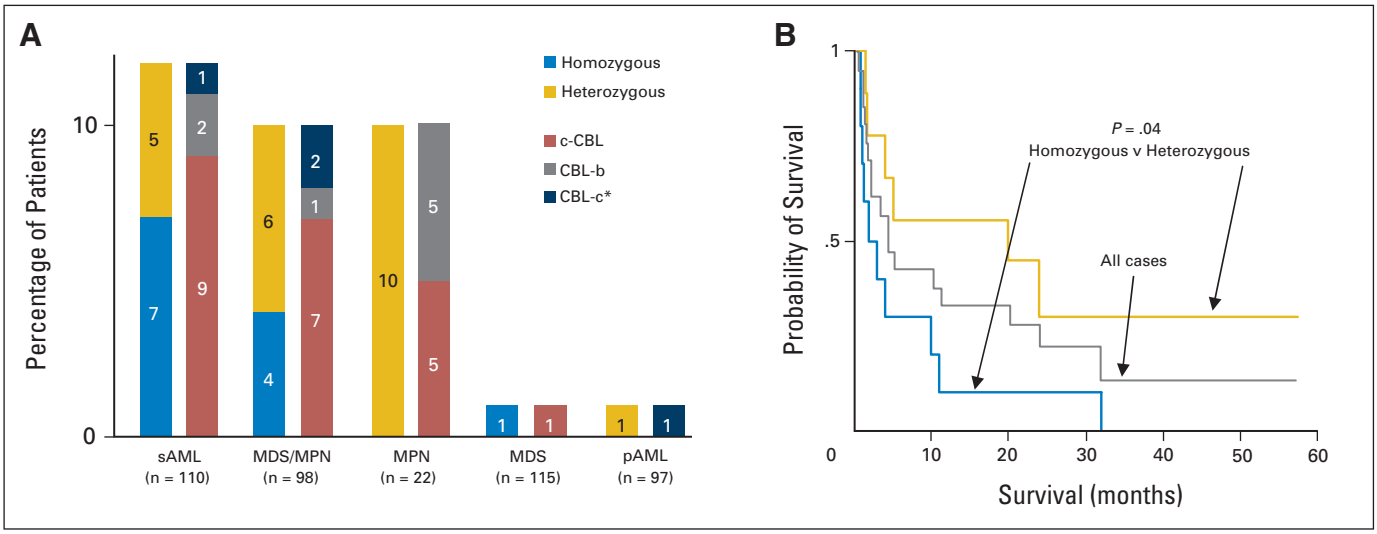

Fig 3. *Cbl* family mutations in myeloid malignancies and unique clinical characteristics of patients with mutations. (A) *Cbl* family mutations are frequently observed in secondary acute myelogenous leukemia (sAML; 12%), myelodysplastic/myeloproliferative neoplasms (MDS/MPN; 10%), and MPN (10%). Homozygous mutations of *c-Cbl* are more frequent in sAML (7%) than MDS/MPN (4%). *Cbl-b* mutations and/or *Cbl-c* frame shift polymorphism (*) are seen in all disease phenotypes. (B) Kaplan-Meier analysis shows overall survival in all cases (n = 20; gray line). Median survival is 5 months and the survival rate is 10%. By comparing patients homozygous for *Cbl* family mutation (n = 10; blue line) to those heterozygous (n = 10; gold line), there is a statistically significant difference in overall survival between these two cohorts regardless of treatment or remission/relapse.

Table 1. Comparison of Clinical Characteristics Between Wild-Type and Mutant *Cbl* Family Genes

Variable*	*Cbl* Family Mutant (n = 17)	*Cbl* Family Wild Type (n = 307)	*P*
Age, years			
≥ 60	10	224	.21
< 60	6	62	
Sex			
Male	8	183	.2
Female	9	106	
WBC count, $\times 10^9$/L			
≥ 10	9	93	.11
< 10	8	197	
Monocyte count, $\times 10^9$/L			
≥ 1	13	53	< .0001
< 1	4	237	
Metaphase cytogetetics			
Abnormal	10	133	.62
Normal	7	139	
Disease risk†			
Advanced grade	12	141	.7
Low grade	4	142	
Therapy and response			
Chemotherapy‡	14	130	.001
Stem cell transplantation	4	21	.035
Complete remission	3	22	.73

*Some clinical data are not available.
†In advanced group, secondary acute myelogenous leukemia, refractory anemia with excess blasts, and chronic myelomonocytic leukemia 2. The others are in low grade group.
‡Chemotherapy includes mitoxantrone, idarubicin, daunorubicin, cytarabine, etoposide, hydroxyurea, fludarabine gemtuzumab, 5-azacitidine, decitabine, lenalidomide, arsenic trioxide, and valproic acid.

Table 2. Univariate Analysis of Overall Survival in Clinical Variables

Variable*	No. of Patients	Overall Survival: Mean (months)	Hazard Ratio	95% CI	*P*
Age, years					
≥ 60	220	32	1.23	0.83 to 1.82	.31
< 60	64	36			
Sex					
Male	176	31	1.37	0.98 to 1.91	.07
Female	108	36			
WBC count, $\times 10^9$/L					
≥ 10	79	25	1.83	1.30 to 2.57	.001
< 10	205	36			
Monocyte count					
≥ 1	48	24	1.79	1.22 to 2.64	.003
< 1	236	35			
Metaphase cytogetetics					
Abnormal	124	30	1.37	0.98 to 1.90	.063
Normal	140	35			
Disease risk†					
Advanced grade	141	18	6.25	4.27 to 9.15	< .0001
Low grade	142	46			
Cbl family gene					
Mutation	16	9	4.64	2.69 to 8.00	< .0001
Wild type	268	34			
Chemotherapy					
Treatment	131	26	2.13	1.54 to 2.93	< .0001
No treatment	153	40			
Stem cell transplantation					
Treatment	24	34	0.87	0.49 to 1.54	.63
No treatment	260	33			
Complete remission					
Yes	23	30	0.70	0.40 to 1.23	.72
No	95	25			

*Some clinical data are not available.
†In advanced group, secondary acute myelogenous leukemia, RAEB, and chronic myelomonocytic leukemia 2. The others are in low-grade group.

as the corresponding gene does not show significant expression in myeloid cells.

Cas-Br-M, a retrovirus, contains *v-Cbl* which corresponds to about one third of the murine *c-Cbl* gene and contains only the murine phosphotyrosine binding domain.[25] This virus consistently induces a type of pre-B cell lymphoma in infected mice. The importance of *c-Cbl* in hematopoiesis has been previously demonstrated in knockout mice that show hyper-responsiveness to hematopoietic growth factors, expansion of the progenitor and stem cell pool, and mild myeloproliferative features.[17] However, recent results obtained with an RFD knock-in in a *c-Cbl*−/− mouse model parallels the phenotype observed in patients; the mutant mouse demonstrated a severe myeloproliferative phenotype (W.Y. Langdon, personal communication). Indeed, in patients mutations were predominantly located in the RFD and affected structurally essential cysteines, possibly led to inactivation of RFD function by frame shift, or created novel splicing sites resulting in larger transcripts. In addition, *c-Cbl* mutations were homozygous or hemizygous, implying that the presence of a WT allele is protective. It is likely that mutations do not lead to the simple knockout of *c-Cbl* function. Rather, by affecting the RFD, they render it a proto-oncogene, consistent with the oncogenic properties of *v-Cbl*. Previously, mutations of *c-Cbl* have been described in a limited number of patients with AML, but neither their function nor their clinical phenotype could be delineated without a comprehensive study of corresponding karyotypes and clinical outcomes.[26-28]

c-Cbl is a member of the *Cbl* family of E3 ubiquitin ligases, which poly- or monoubiquitinate a number of important tyrosine kinases serving as important transduction elements of proliferative signals and activated tyrosine kinase receptors, including Flt-3, c-kit, and M-CSF.[29,30] Consequently, inactivation of ubiquitination may lead to enhanced and prolonged signaling, a function which can explain the phenotype in patients (Fig 4). Based on this essential role of E3 ligases, we hypothesized that another *Cbl* family member, *Cbl-b*, may also be affected by mutations in myeloid malignancies. Sequencing of these genes in patients who did not harbor *c-Cbl* mutations revealed that

Table 3. Multivariate Analysis of Overall Survival in Clinical Variables

Variable*	Hazard Ratio	95% CI	*P*
Disease risk (advanced grade/ low grade)	5.64	3.8 to 8.36	< .0001
Cbl family gene (mutation/ wild type)	2.17	1.18 to 4.02	.013

*WBC, monocyte count, disease risk, chemotherapy, and *Cbl* family mutation were included in multivariate analysis.

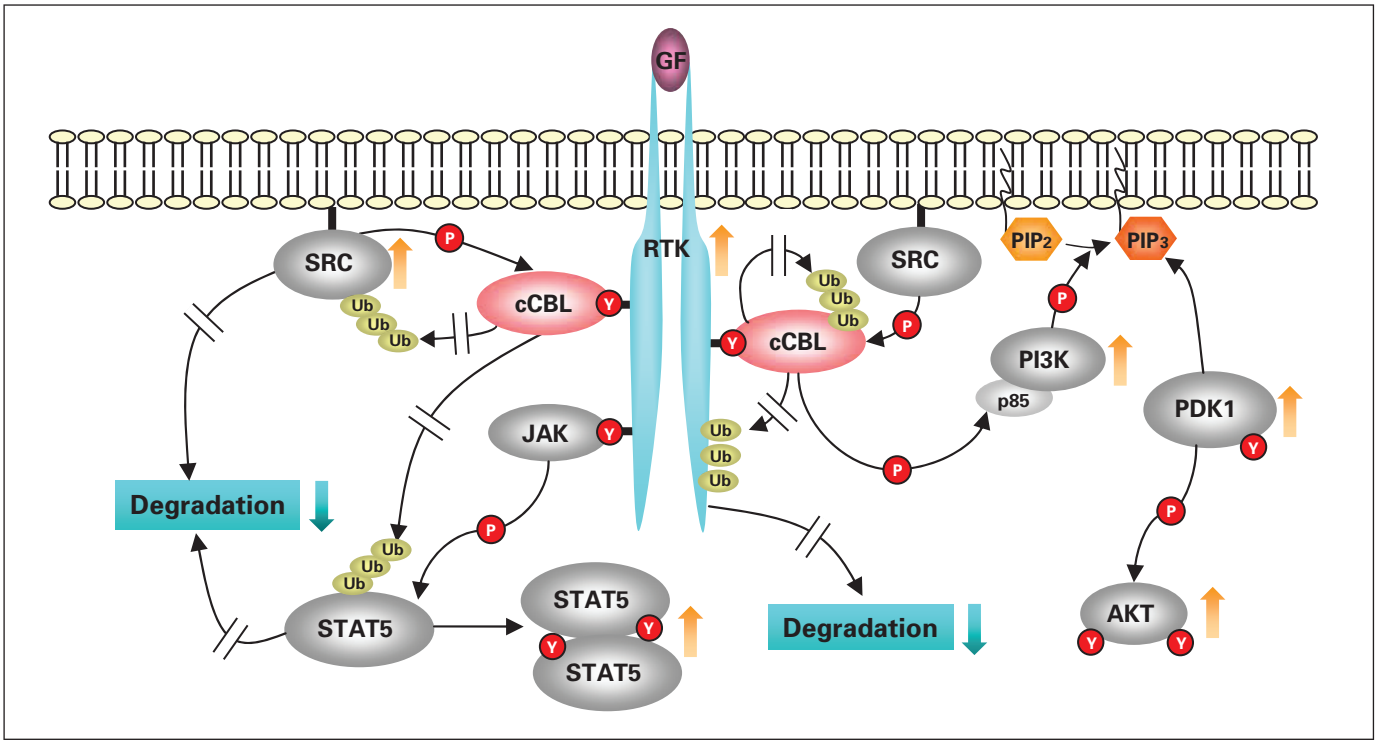

Fig 4. Potential intracellular consequences of *c-Cbl* mutations. *c-Cbl* is a member of the E3 ubiquitin ligase *Cbl* family, which poly- or monoubiquitinate a number of important receptor tyrosine kinases (RTK), including Flt-3, c-kit, and CSF-1, for degradation. Inactivation of ubiquitination activity through mutations occurring in RING finger domain may lead to enhanced and/or prolonged hematopoietic growth factor signaling, a function which can contribute to the clinical phenotype of patients with *c-Cbl* mutation. However, in addition to RTK, knockout of c-Cbl ubiquitination activity may result in enhanced phosphorylation of SRC kinase, STAT5, and also c-Cbl itself. Ultimately, the elevated level of important transduction factors such as STAT5 and PI3K as well as increased SRC kinase activity can lead to aberrant proliferative responses.

these genes can also be affected by mutations leading to the inactivation of the RFD. These patients displayed a clinical phenotype analogous to those with *c-Cbl* mutations. The clinical features corresponding to *c-Cbl* mutations included monocytic features, aberrant and increased phosphorylation of pSTAT5, and monocytoid blasts. An increased frequency of mutations in patients with frank AML may argue either that *c-Cbl* mutations lead to an invariant progression to an aggressive phenotype, or that they constitute a second hit event frequently occurring in the context of atypical myeloproliferative disorders. In fact, unlike other reports that looked at patients with inv16, we found no mutation of *Cbl* family genes in de novo AML, including French-American-British type M4 or M5.[28] Both theories are supported by the dismal prognosis of patients with *Cbl* family mutations. The close association of *c-Cbl* mutations with monocyte expansion, such as that seen in JMML, CMML, or sAML with monocytoid features, suggests a primary role of *c-Cbl* mutations in the pathogenesis of these diseases, while occurrence of *c-Cbl* mutations during evolution to AML in serially studied patients and the high proportion of cases with advanced leukemia affected by *c-Cbl* mutations argues for its auxiliary facilitator role.

Taken together, our data suggests that *Cbl* family mutations constitute a novel class of pathogenic molecular lesions associated with a spectrum of myeloid malignancies characterized by myeloproliferative features and poor prognosis. Inactivation of the RFD, and thereby ubiquitination involved in downmodulation of proliferative signaling, constitutes a general mechanism of leukemogenesis likely present in a variety of malignancies.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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