Mutations of an E3 ubiquitin ligase *c-Cbl* but not *TET2* mutations are pathogenic in juvenile myelomonocytic leukemia

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Juvenile myelomonocytic leukemia (JMML) is a rare pediatric myeloid neoplasm characterized by excessive proliferation of myelomonocytic cells. When we investigated the presence of recurrent molecular lesions in a cohort of 49 children with JMML, neurofibromatosis phenotype (and thereby *NF1* mutation) was present in 2 patients (4%), whereas previously described *PTPN11*, *NRAS*, and *KRAS* mutations were found in 53%, 4%, and 2% of cases, respectively.

Consequently, a significant proportion of JMML patients without identifiable pathogenesis prompted our search for other molecular defects. When we applied single nucleotide polymorphism arrays to JMML patients, somatic uniparental disomy 11q was detected in 4 of 49 patients; all of these cases harbored RING finger domain *c-Cbl* mutations. In total, *c-Cbl* mutations were detected in 5 (10%) of 49 patients. No mutations were identified in *Cbl-b* and *TET2*.

***c-Cbl* and *RAS* pathway mutations were mutually exclusive. Comparison of clinical phenotypes showed earlier presentation and lower hemoglobin F levels in patients with *c-Cbl* mutations. Our results indicate that mutations in *c-Cbl* may represent key molecular lesions in JMML patients without *RAS/PTPN11* lesions, suggesting analogous pathogenesis to those observed in chronic myelomonocytic leukemia (CMML) patients. (Blood. 2010;115:1969-1975)**

Introduction

Juvenile myelomonocytic leukemia (JMML) is a special subtype of myelodysplastic syndrome/myeloproliferative disorder (MDS/MPD) that, analogous to chronic myelomonocytic leukemia (CMML), is characterized by excessive proliferation of myelomonocytic cells, but unlike CMML it occurs in young children and shows characteristic hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF).[1-3] Mutations of genes involved in GM-CSF signal transduction, including *RAS* and *PTPN11*, can be identified in a majority of children with JMML.[3-5] Constitutional mutations of *NF1* can be found in another 10% of patients with JMML.[1,6,7] Recent studies show that a common mechanism of *NF1* inactivation is uniparental disomy (UPD) resulting in duplication of the mutant *NF1* allele.[7,8] *NF1* is a GTPase activating protein for RAS and thereby acts as a tumor suppressor.[9] Oncogenic *RAS* mutations at codons 12, 13, and 61 have been identified in approximately 20% to 25% of patients with JMML.[4,10] These mutations lead to elevated levels of RAS-GTP, the active form of RAS.[11] Somatic mutations in *PTPN11*, coding for tyrosine phosphatase Src homology 2 domain-containing protein, have been reported in 35% of patients with JMML,[5,12,13] and induce hematopoietic progenitor hypersensitivity to GM-CSF due to hyperactivation of the RAS signaling axis.[14,15]

Based on the proposed paradigm that recurrent areas of somatic copy-neutral loss of heterozygosity can point toward the presence of homozygous mutations contained within the corresponding region,[16] we have identified various recurrent areas of acquired segmental UPD, in particular in patients with MDS/MPD, including CMML. Such analyses have shown that, in addition to the recently identified *Jak2V617F* mutation associated with UPD9p, other known mutations can be duplicated by homologous recombination, including, for example, *c-Mpl* (UPD1p), *FLT-3* ITD (UPD13q), *TET2* (UPD4q), and others.[17-22] Based on the observation of recurrent somatic UPD11q23.3, we have discovered homozygous *c-Cbl* mutations in the RING finger domain (RFD) occurring frequently in MDS/MPD and especially CMML or secondary acute myeloid leukemia (AML) evolved from CMML.[23] When we analyzed other members of *Cbl* gene family, mutations were also found in *Cbl-b* and *Cbl-c* and were associated with an indistinguishable clinical phenotype.[24] The *Cbl* gene family codes for E3 ubiquitin ligases (ULs) with the ubiquitination activity mediated via the RFD. They are involved in degradation of activated phosphotyrosine receptors and other phosphotyrosine kinases such as ζ-chain–associated protein kinase 70 involved in signal transduction.[25] Thus, mutations in the RFD can lead to decreased receptor degradation and, analogous to *PTPN11* mutations, result in augmentation of proliferative signals mediated by various growth factor receptors. In a *c-Cbl*^{−/−} mouse model a mild myeloproliferative phenotype with expansion of stem cells and hyperresponsiveness to growth factors is found,[26] whereas a RFD mutant knock-in model shows a severe myeloproliferative phenotype (W. Langdon, University of Western Australia, oral communication, January 2009). These observations, together with the transforming effects of the

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v-Cbl oncogene lacking the RFD, suggest that E3 UL activity is essential for the tumor suppressor function of *c-Cbl*, whereas the N-terminal portion of the protein may be oncogenic.

Based on the morphologic similarities of JMML and typical CMML, presence of growth factor hypersensitivity, and observation of UPD11q in children affected by JMML, we hypothesized that *Cbl* family mutations may also be present in a subset of patients with JMML. Here, we investigated 49 JMML patients with the goals of (1) identifying pathogenic molecular lesions, including mutations in *Cbl* gene family members, and (2) correlating clinical outcomes to presence and location of other pathogenic molecular lesions, including *PTPN11*, *NRAS*, *KRAS*, and *TET2*. Of note is that during review of our paper, *c-Cbl* mutations were reported in JMML.[27]

Methods

Patients

We studied 49 children (32 boys and 16 girls; 1 patient's sex was unknown) with JMML diagnosed between 1988 and 2008 in 28 institutions throughout Japan. Written informed consent for sample collection was obtained at appropriate institutions from patients' parents according to the institutional protocols and the Declaration of Helsinki. The sample repository was located at Nagoya University Graduate School of Medicine. Molecular analysis of the mutational status was approved by the Ethics Committee of Nagoya University Graduate School of Medicine. The diagnosis of JMML was based on the internationally accepted criteria previously published.[26] We excluded patients with Noonan syndrome. The clinical and hematologic characteristics of the patients are summarized in Table 1. The median age at diagnosis was 28 months (range, 1-75 months). Karyotypic abnormalities were detected in 11 patients, including 7 patients with monosomy 7. Two children had clinical evidence of *NF1*. Of 49 patients, 32 underwent hematopoietic stem cell transplantation.

Table 1. Characteristics of JMML patient cohort

Variable	Total cohort, N = 49
Median age at diagnosis, mo (range)	32 (1-75)
Sex, male/female/unknown	32/16/1
NF1 by clinical diagnosis, yes/no	2/47
Median Hb, g/L (range)	0.96 (0.49-1.20)
Median HbF, % (range)	23.6 (1.0-62.0)
Median WBC, $\times 10^9$/L (range)	28.0 (10.9-126.2)
Median monocyte in PB count, $\times 10^9$/L (range)	4.5 (1.0-31.6)
Median plt, $\times 10^9$/L (range)	49 (1.4-320)
Metaphase cytogenetics, no. of patients (%)	
Normal karyotype	35 (71.4)
Monosomy 7	8 (16.3)
Trisomy 8	1 (2.0)
Other abnormalities	3 (6.1)
Unknown	2 (4.1)
Hematopoietic stem cell transplantation	
Yes	32
No	13
Unknown	4
Status at last follow-up	
Alive	24
Dead	21
Unknown	4
Median observation period, mo (range)	14 (1-216)

NF1 indicates neurofibromatosis type1; Hb, hemoglobin; WBC, white blood cell; PB, peripheral blood; and plt, platelet.

SNP-A karyotyping analysis

Mononuclear cells were isolated using Ficoll-Hypaque density gradient centrifugation and cryopreserved until use. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN). High-density Affymetrix single nucleotide polymorphism array (SNP-A; 250 K) was applied as a karyotyping platform to identify loss of heterozygosity (LOH), microamplification, and microdeletion as previously described.[28]

Bioinformatic analysis

Signal intensity was analyzed and SNP calls were determined using Gene Chip Genotyping Analysis Software Version 4.0 (GTYPE). Copy number and areas of UPD were investigated using a Hidden Markov Model and CN Analyzer for Affymetrix GeneChip Mapping 250-K arrays (CNAG Version 3.0) as previously described.[28]

We excluded germline-encoded copy number variation and nonclonal areas of gene copy number–neutral LOH from further analysis using a bioanalytic algorithm based on lesions identified by SNP-A in an internal control series (N = 713) and reported in the Database of Genomic Variants (http://projects.tcag.ca/variation).[29] Through calculation of their average sizes, we defined a maximal size of germline LOH in controls and consequently excluded all defects of this type in patients' samples; according to 95% confidence interval, stretches of UPD larger than 25.8 Mb were considered unlikely of germline origin. In addition, all nonclonal areas of UPD seen in controls were interstitial.

PTPN11, *NRAS*, *KRAS*, *TET2*, and *E3* ubiquitin ligase mutational screening

To screen for *PTPN11* mutations, we polymerase chain reaction amplified genomic DNA corresponding to exons 2, 3, 4, 7, 8, 12, and 13 as previously reported.[12,30,31] *NRAS* and *KRAS* mutations in codons 12, 13, and 61 were identified as previously described[32,33] and were confirmed by sequencing. To screen patients for mutations in E3 ubiquitin ligase genes and *TET2*, direct genomic sequencing of exons constituting the RFD of *Cbl* family members (exons 8 and 9 of *c-Cbl*, exons 9 and 10 of *Cbl-b*, exons 7 and 8 of *Cbl-c*, and exons 3-11 of *TET2*) was performed. For sequencing, 250 ng of polymerase chain reaction product, 3μM original forward or reverse primer, 2 μL of Big Dye Version 3.1 (Applied Biosystems), and 14.5 μL of 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.[22]

GM-CSF hypersensitivity assay

GM-CSF hypersensitivity assays were established as described previously.[2] Briefly, we used cytokine-free methocult H4230 (StemCell Technologies), and added 1×10^3 CD34$^+$ bone marrow cells that were prepared by positive selection with magnetic-activated cell sorting beads (Miltenyi Biotec). Recombinant human GM-CSF (R&D Systems) was added at the time the cultures were initiated. Cultures were performed in duplicate, and colonies of 40 or more cells were scored after 14 days of incubation. The data are expressed as percentage of maximal numbers of granulocyte-macrophage colony-forming units (CFU-GMs). This approach more accurately reflects changes in sensitivity and does not bias the results compared with graphing actual counts because most JMML samples had considerably higher total numbers of CFU-GMs than controls, although there was considerable patient-to-patient variability.

Statistical analysis

When appropriate, Kaplan-Meier statistics were applied to assess survival. For comparison of the frequency of mutation or other clinical features between disease groups, categoric variables were analyzed using the Fisher exact test and continuous variables were tested using the Mann-Whitney *U* test.
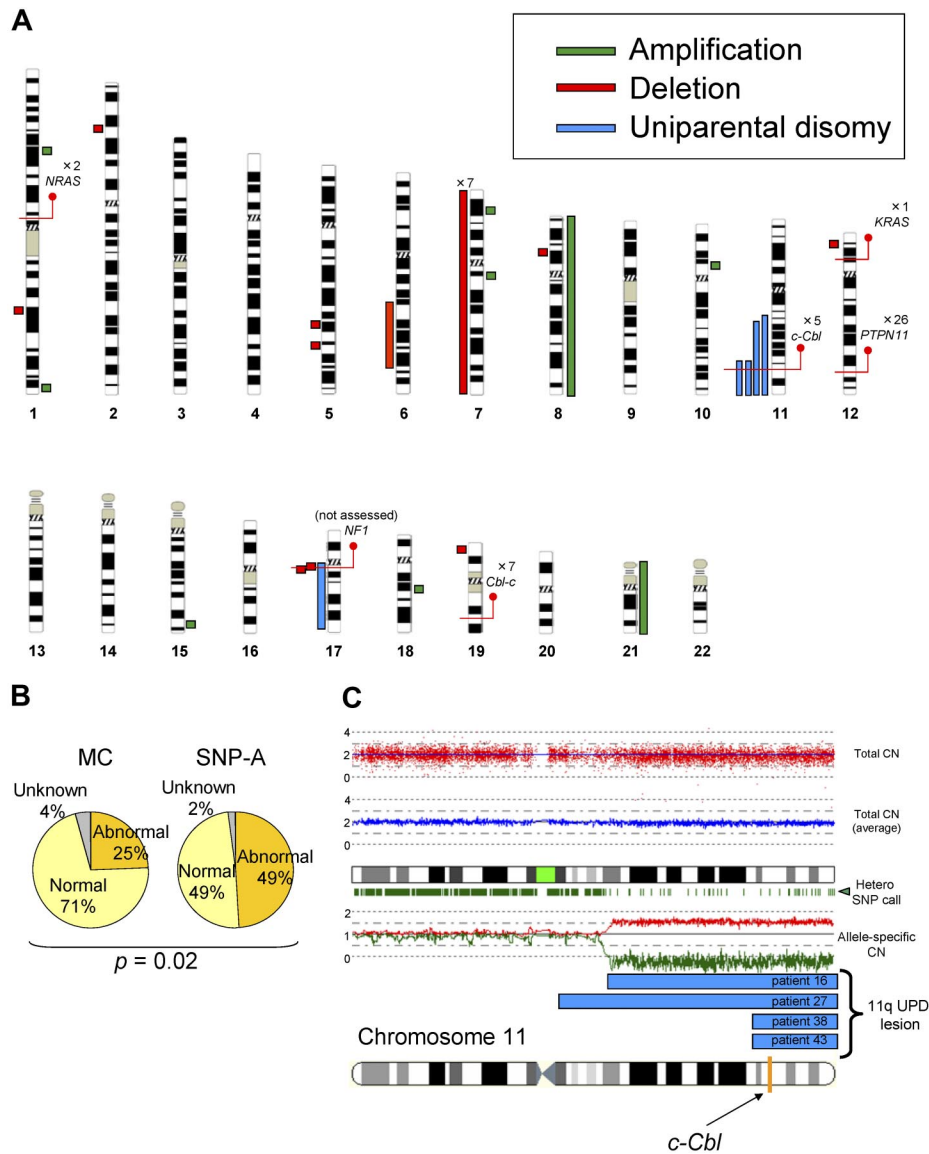
Figure 1. Single nucleotide polymorphism array–based karyotyping of JMML. (A) Genomic distribution and type of lesion identified in patients with JMML by SNP-A analysis. Green bar represent amplification, red shows deletion, and blue corresponds to UPD. Red lines pinpoint the locus of genes discussed in the text, as well as the number of patients mutated at that locus. *NF1* mutational status was not assessed in this cohort. (B) Increased sensitivity of SNP-A for detecting chromosomal lesions. The results of MC (25%) and by SNP-A (49%) from the JMML cohort studied are shown. (C) Representative 250-K SNP-A analysis of UPD11q by CNAG Version 3.0 (patient 16). Both the raw and averaged total copy number (CN) tracks (red dots, blue line) show a normal copy number, whereas heterozygous SNP calls and allele-specific copy number tracks (green dashes, red/green lines) show a reduction in copy number, indicating UPD. The specific localization of 11qUPD in 4 patients (patients 16, 27, 38, and 43) is indicated by the blue bars. The *c-Cbl* locus is indicated on the chromosome 11 idiogram with a yellow line.

Results

Cytogenetic and clinical characterization of JMML patients

First, we performed SNP-A– and metaphase cytogenetics–based analyses. Using conventional metaphase cytogenetics, chromosomal aberrations were found only in a minority of patients (25%). SNP-A–based karyotyping confirmed the results of metaphase cytogenetics, including the presence of monosomy 7 in 7 patients and trisomy 8 in 1 patient. However, due to increased precision and ability to detect copy-neutral loss of heterozygosity of SNP-A, additional lesions were identified by SNP-A in 24 (49%) of 48 patients, including trisomy 21 not detected by metaphase cytogenetics (MC) in 1 patient and microdeletions in 9 patients (Figure 1A), including 1q25.3 (patient 44), 2p22.1 (patient 46), 5q23.1 (patient 20), 5q31.3 (patient 40), 6q21q25.3 (patient 15), 8p21.2 (patient 36), 12p13.2 (patient 3), 17q11.2 (patients 15, 49), and

19p13.3 (patient 2). We also detected microamplifications in 7 patients (Figure 1A), located at 1p31.1 (patient 14), 1q44 (patient 39), 7p21.1 (patient 16), 7q11.22 (patient 17), 10p11.23 (patient 29), 15q26.3 (patient 47), and 18q12.3 (patient 22). The shared copy number–altering lesions included monosomy 7 and loss of 17q11.2, which contained the *NF1* locus. Although we were unable to confirm the somatic nature of the submicroscopic defects due to lack of germline DNA, these lesions did not overlap with copy number variations present in internal control cohort and publicly available databases. Most significantly, we identified UPD in 5 patients (Figure 1A). UPD11q was found in 4 patients, all regions overlapping from 11q23.3 to the telomere. This commonly affected region contained the *c-Cbl* locus (Figure 1A,C). The region of UPD at 17q contained the *NF1* locus and corresponded with clinical neurofibromatosis features. Overall, compared with the results of MC, SNP-A identified significantly more genetic abnormalities (25% vs 49%; *P* = .02; Figure 1B).

Table 2. Patients' mutational status

Patient number	PTPN11	NRAS	KRAS	c-Cbl	Cbl-b	TET2
1	■					
2	■					
3						
4						
5						
6						
7	■					
8	■					
9	■					
10	■					
11						
12						
13	■					
14	■					
15						
16				■		
17	■					
18	■					
19						
20						
21	■					
22	■					
23				■		
24	■					
25			■			
26		■				
27				■		
28	■					
29	■					
30		■				
31	■					
32	■					
33						
34						
35						
36	■					
37	■					
38				■		
39	■					
40	■					
41	■					
42	■					
43				■		
44						
45	■					
46						
47	■					
48						
49	■					
Total	**26**	**2**	**1**	**5**	**0**	**0**

Gray cells represent mutation; and white cells, wild type.

Mutational analysis of patients with JMML

After defining chromosomal defects associated with JMML, we performed mutational analysis of the genes known to be affected by mutations in JMML. *PTPN11* mutations were found in 26 (53%) of 49, whereas *NRAS* and *KRAS* mutations were found in 2 (4%) of 49

and 1 (2%) of 49, respectively (Table 2). None of the patients screened show the presence of *TET2* mutations, previously shown to be present in a significant proportion of patients with MDS/MPD, including CMML.[21] Excluding patients with a neurofibromatosis phenotype, 18 (37%) of 49 of patients did not show any of the known pathogenic defects occurring in JMML.

Identification of *Cbl* gene family mutations in JMML

Previously, homozygous *c-Cbl* mutations in the RFD were identified in patients with MDS/MPD, especially CMML or secondary AML that evolved from CMML.[22] We focused our attention on this gene as UPD11q was found in 4 of 49 JMML patients. Mutational analysis of *Cbl* family genes revealed mutations of *c-Cbl* in 5 (10%) of 49 patients, and no *Cbl-b* mutations (Tables 2-3). *c-Cbl* mutations were heterozygous in 1 patient (patient 23) and homozygous in 4 patients (patients 16, 27, 38, 43; supplemental Table 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). All mutations were located in the RFD (exon 8 and intron 8); 2 patients had an identical homozygous mutation (1111T>C, Tyr371His; patients 27, 38). All 4 patients with a homozygous *c-Cbl* mutation simultaneously harbored UPD11q (supplemental Table 1, Figures 1C-2). In addition, no patient with a *c-Cbl* mutation had mutations in genes known to play a role in JMML (*PTPN11*, *NRAS*, and *KRAS*; Table 2) or had clinical diagnosis of NF1. Excluding patients with a neurofibromatosis phenotype, 13 (26.5%) of 49 of patients did not have the mutation of *PTPN11*, *RAS*, and *c-Cbl* genes.

Table 3. Summary of *c-Cbl* mutations in patients with JMML

Patient number	Pherograms of sequence	*c-Cbl* mutation			
		Homo /Hetero	Nucleotide change	Amino acid change	Location
16		Homo	1202 G>C	Cys401Ser	Exon 8
23		Hetero	1106 del 66bp	Deletion	Exon 8
27		Homo	1111 T>C	Tyr371His	Exon 8
38		Homo	1111 T>C	Tyr371His	Exon 8
43		Homo	1228-2 A>G	Splice acceptor site	Intron 8

Figure 2. Site of the *c-Cbl* mutations and predicted product of splice variant in the intron 8 splice acceptor site. (A) Localization of the c-Cbl mutations within the predicted protein product. Red arrows show the site of mutations in exon, and blue arrows show the site of splice variant. (B) In patient 48, a homozygous mutation was seen in the intron 8 splice acceptor site of *c-Cbl*. According to http://genome.cbs.dtu.dk/services/NetGene2,[34] this mutation may result in a splice variant, leading to a shorter transcript in RF domain.
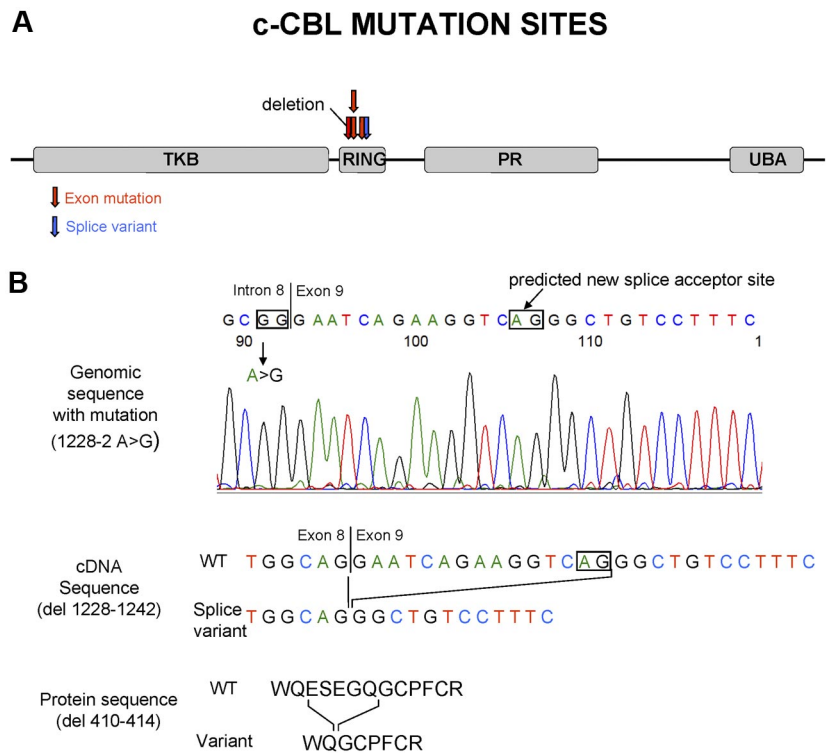
When we investigated our cohort for the presence of *Cbl-c* mutations, we found heterozygous frameshift nucleotide variation (1256 insertion C; patients 7, 12, 14, 29, 33, 38, and 46; data not shown). However, *Cbl-c* mutational status of germline though sequencing of nonclonal CD3$^+$ lymphocytes in those patients showed the same frameshift mutation. Consequently, these *Cbl-c* nucleotide exchanges represent rare polymorphisms.
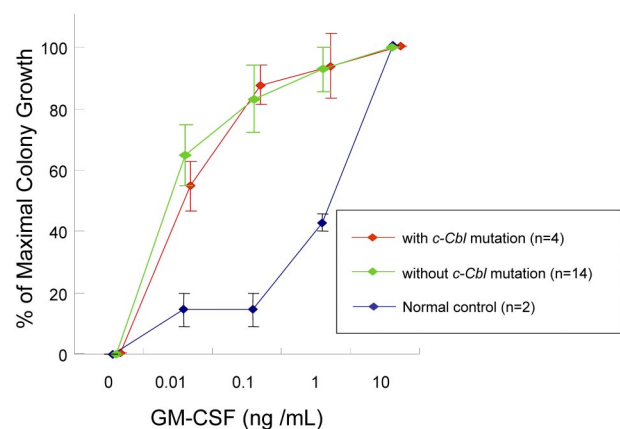
GM-CSF hypersensitivity assay

We also investigated whether JMML-specific GM-CSF is related exclusively to individual types of mutations identified, including c-Cbl mutations. CD34$^+$ bone marrow cells' colony counts are expressed as percentage of maximal (supraoptimal) number of CFU-GMs (colony counts at any given concentration of GM-CSF/ colony counts at 10 ng/mL GM-CSF). The colony growth of JMML cells with or without *c-Cbl* mutation did not differ from normal controls in low concentration of GM-CSF. For example at 0.01 ng/mL GM-CSF, colony counts were 55% ($\pm$ 8%) with *c-Cbl* mutation (n = 4) versus 65% ($\pm$ 10%) without *c-Cbl* mutation (n = 14) versus 15% ($\pm$ 5%) in controls (n = 2; *P* = .042). At 0.1 ng/mL GM-CSF, colony counts were 87% ($\pm$ 6%) versus 83% ($\pm$ 11%) versus 15% ($\pm$ 5%); *P* = .011) and at 1.0 ng/mL GM-CSF, 94% ($\pm$ 11%) versus 93% ($\pm$ 7%) versus 43% ($\pm$ 3%; *P* = .063), respectively. Consequently, our results indicate that GM-CSF hypersensitivity of CD34$^+$ cells from JMML patients may be a result of various molecular lesions including *c-Cbl* mutations (Figure 3).

Clinical features associated with *Cbl* gene family mutations

Although different molecular lesions can result in similar clinical phenotypes, specific mutations can modify clinical behavior and morphologic features. Consequently, we analyzed clinical characteristics of patients with specific mutations (Table 4).

We did not find any distinctive morphologic features of patients with *Cbl* gene family mutations and no differences were present in

the blood counts at initial presentation. Other variables studied (sex, the presence of cytogenetic abnormalities) also did not differ between patients grouped according to mutational status. However, patients with mutant *c-Cbl* compared with those with wild-type constellation showed earlier presentation (median age at diagnosis, 12 months vs 29 months, *P* = .037) and lower median hemoglobin F (HbF) percentage (3.5% vs 24.9%, *P* = .02), previously shown to correlate with less favorable prognosis.[1,33,35-40] Low HbF values in *c-Cbl* mutant cases were not attributable to monosomy 7, absent in this patient cohort. The probability of 2-year overall survival of *c-Cbl* mutant patients (50.0%; 95% confidence interval [CI], 25.0%-75.0%; n = 4) was similar to that of patients without *c-Cbl* mutations (50.4% [95% CI, 42%-59%]; n = 41). Similarly, when

Figure 3. GM-CSF hypersensitivity assay. Colony counts are expressed as percentage of maximal numbers of CFU-GM (colony counts cultured with each concentration of GM-CSF/colony counts cultured with 10 ng/mL GM-CSF). The similar GM-CSF hypersensitivity was seen in JMML patients with or without *c-Cbl* mutation. Error bars represent SE.

Table 4. Comparison of clinical characteristics for JMML patients with and without *c-Cbl* mutation

Variable	With *c-Cbl* mutation, n = 5	Without *c-Cbl* mutation, n = 44	P
Median age at diagnosis, mo (range)	12 (8-15)	29 (1-75)	.037
Median HbF, % (range)	3.5 (2.0-7.6)	24.9 (1.0-62.0)	.02

Other variables studied (sex, hemoglobin level, white blood cell count, platelet count, monocyte percentage in peripheral blood, and metaphase cytogenetic abnormalities) do not show statistical significance.

HbF indicates hemoglobin F.

patients with all *Cbl* gene family mutations were analyzed, no distinct clinical features including differences in outcomes were found.

Discussion

The molecular pathogenesis of the often heterogeneous myeloid malignancies is not discernable through traditional morphologic analyses. Conversely, various molecular mechanisms can lead to similar clinical phenotypes and distinct mutational steps can result in various types of functional defects, each requiring distinct therapeutic approaches. Although JMML is associated with mutations in *PTPN11* and *RAS* in a large proportion of cases[3-5,12,13] and mutations of *NF-1* in a smaller fraction,[1,6,7] no specific mutations can be identified in a number of children affected by this disease.

Previously, we identified UPD11q and associated homozygous *c-Cbl* mutations in patients with CMML and secondary AML with monocytoid features.[23] We have also noted that heterozygous mutations of other closely related E3 ULs such as *Cbl-b* and *Cbl-c* may be found in some patients with otherwise indistinguishable morphologic features; these mutations presented in heterozygous constellation as they were not associated with corresponding areas of somatic UPD.[24] We have also found a significant proportion of CMML cases with UPD4q and microdeletions corresponding to the location of *TET2* gene. We have shown that UPD4q is associated with *TET2* mutations but, unlike for *c-Cbl,* heterozygous *TET2* mutations were common.[22]

Based on our progress in CMML, in this article we undertook the molecular analysis of cytogenetic abnormalities and mutational events in the clinically similar syndrome of JMML occurring in children. Using SNP-A analysis we show that patients with JMML, in addition to known typical chromosomal defects, harbor invariant somatic copy-neutral loss of heterozygosity, in particular UPD11q23.3. Based on this finding and the previously shown association of UPD11q with *c-Cbl* mutation, we demonstrated that *c-Cbl* mutations located in the RFD of this gene are found in 5 (10%) of 49 of JMML patients. Since submission of this paper, similar results were reported by Loh et al.[27] Unlike in adult CMML, *TET2* mutations were not identified in JMML, a finding consistent with the absence of UPD4q or del4 in JMML.

Our findings suggest that selective pressure in JMML leads to use of functionally related pathways but may involve distinct genes. In fact, both *c-Cbl* (ubiquitination) and *PTPN11* (dephosphorylation) mutations can lead to the augmentation of growth factor receptor–mediated signals and may explain why GM-CSF hypersensitivity is present in patients with JMML irrespective of whether *c-Cbl, PTPN11,* or *RAS* is mutated.

For *Cbl* mutations, in addition to the impaired degradation of activated growth factor receptors, altered ζ-chain–associated protein kinase 70 activation by c-Cbl may mediate proliferative signals analogous to RAS. Moreover, by binding to Grb2, c-Cbl competes with the guanine-nucleotide-exchange factor son-of-sevenless, thereby blocking signaling through the RAS–mitogen-activated protein kinase pathway and inhibiting proliferation.[25] In agreement with this theory, RFD mutant knock-in mouse experiments suggest that *c-Cbl* deprived of its E3 ligase activity may act as an oncogene, and functional analysis of mutated *c-Cbl* showed that mutated *c-Cbl* has an oncogenic effect.[35] These findings conclusively prove the pathogenic role of *c-Cbl* mutation in hematologic malignancies.

Our earlier studies showed that *c-Cbl* mutations stem from a somatic event and are not present in germline[23]; however, germline DNA was not available from our patients to conduct confirmatory studies. Nevertheless, *c-Cbl* mutations in JMML were similar or identical to those previously shown in CMML, for which the somatic nature has been confirmed through analysis of germline DNA and serial studies. Similarly, *c-Cbl* mutations were present exclusively in the context of UPD11q23.3, shown to occur only as a clonal somatic event. In agreement with a previous report,[7] we have also found UPD17q in association with neurofibromatosis-associated JMML.

Patients with *c-Cbl* mutations show comparable survival as those without *c-Cbl* mutations, but a large fraction of these patients underwent transplantation. However, *c-Cbl* mutations were associated with a younger age of presentation and smaller percentage of HbF. Given that in previous reports an older age at diagnosis and elevated HbF level have been repeatedly described as risk factors for survival in JMML,[1,36-42] lack of these poor prognostic markers in *c-Cbl* patients who demonstrate a similar outcome argues for an unfavorable impact of *c-Cbl* mutation, analogous to adult patients with *c-Cbl*.

In summary, our study describes a novel molecular lesion in children affected by JMML, suggesting similarity in the pathogenesis of a portion of patients with JMML to those with CMML.

Authorship

Contribution: H. Muramatsu and H. Makishima designed research, performed research, analyzed data, and wrote the paper; A.M.J. and H.C. performed research; C.O. designed research, analyzed data, and wrote the paper; N.Y., Y.X., N.N., A.H., H.Y., Y.T., K.K., and A.M. designed research; S.K. designed research and wrote the paper; and J.P.M. designed research, performed research, analyzed data, and wrote the paper.

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