Molecular and phenotypic diversity of *CBL*-mutated juvenile myelomonocytic leukemia

Anna Hecht,[1,2] Julia A. Meyer,[1] Astrid Behnert,[1] Eric Wong,[1] Farid Chehab,[3] Adam Olshen,[4,5] Aaron Hechmer,[4] Catherine Aftandilian,[6] Rukhmi Bhat,[7] Sung Won Choi,[8] Satheesh Chonat,[9] Jason E. Farrar,[10] Mark Fluchel,[11] Haydar Frangoul,[12] Jennifer H. Han,[13] Edward A. Kolb,[14] Dennis J. Kuo,[13] Margaret L. MacMillan,[15] Luke Maese,[16] Kelly W. Maloney,[17] Aru Narendran,[18] Benjamin Oshrine,[19] Kirk R. Schultz,[20] Maria L. Sulis,[21] David Van Mater,[22] Sarah K. Tasian,[23] Wolf-Karsten Hofmann,[2] Mignon L. Loh[1,4] and Elliot Stieglitz[1,4]

[1]Department of Pediatrics, Benioff Children's Hospital, University of California San Francisco, San Francisco, CA, USA; [2]Department of Hematology/Oncology, University Hospital Mannheim, Heidelberg University, Heidelberg, Germany; [3]Department of Laboratory Medicine, University of California San Francisco, San Francisco, CA, USA; [4]Helen Diller Family Comprehensive Cancer Center, University of California San Francisco, San Francisco, CA, USA; [5]Department of Epidemiology and Biostatistics, University of California San Francisco, San Francisco, CA, USA; [6]Pediatric Hematology/Oncology, Stanford University, Stanford, CA, USA; [7]Northwestern University Feinberg School of Medicine, Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, IL, USA; [8]Blood and Marrow Transplantation Program, University of Michigan, Ann Arbor, MI. USA; [9]Department of Pediatrics, Emory University School of Medicine, Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta, Atlanta, GA, USA; [10]Arkansas Children's Research Institute, Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, AR, USA; [11]University of Utah, Department of Pediatrics, Division of Pediatric Hematology-Oncology, Salt Lake City, UT, USA; [12]The Children's Hospital at TriStar Centennial and Sarah Cannon Research Institute, Nashville, TN, USA; [13]Division of Pediatric Hematology-Oncology, University of California San Diego/Rady Children's Hospital San Diego, CA, USA; [14]Nemours Center for Cancer and Blood Disorders/Alfred I. DuPont Hospital for Children, Wilmington, DE, USA; [15]Blood and Marrow Transplant Program, Department of Pediatrics, University of Minnesota Medical School, Minneapolis, MN, USA; [16]Department of Pediatrics and Huntsman Cancer Institute, University of Utah, Salt Lake City, UT, USA; [17]Children's Hospital Colorado, Aurora, CO, USA; [18]Pediatric Hematology and Oncology, Alberta Children's Hospital, Calgary, Alberta, Canada; [19]Johns Hopkins All Children's Hospital, St. Petersburg, FL, USA; [20]British Columbia Children's Hospital and Research Institute, Vancouver, British Columbia, Canada; [21]Department of Pediatrics, Memorial Sloan Kettering Cancer Center, New York, NY, USA; [22]Department of Pediatrics, Duke University Medical Center, Durham, NC, USA and [23]Division of Oncology and Center for Childhood Cancer Research, Children's Hospital of Philadelphia; Department of Pediatrics and Abramson Cancer Center, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA.

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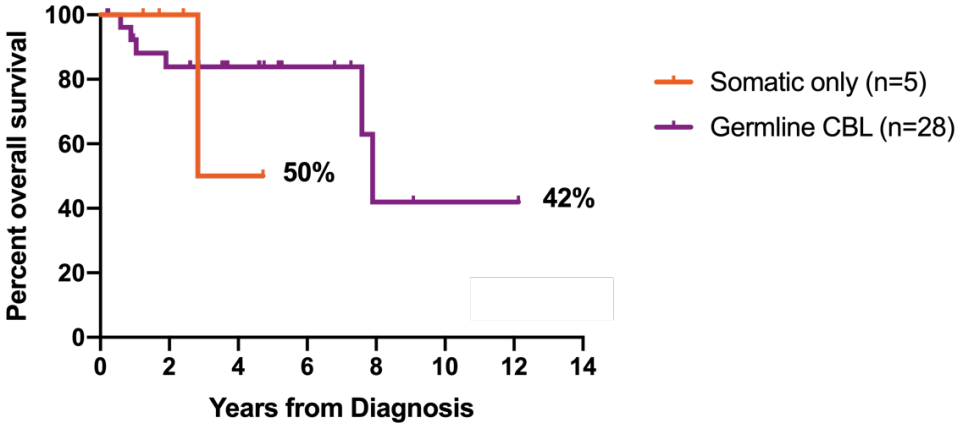
Correspondence: *ELLIOT STIEGLITZ* - elliot.stieglitz@ucsf.edu

Supplemental Material for Manuscript "Molecular and phenotypic diversity of *CBL*-mutated juvenile myelomonocytic leukemia":

Supplementary Table 1. Genes covered in our custom JMML amplicon next-generation sequencing panel. Sequencing either covered the full length of the gene ("full") or hotspot locations of known mutations ("hotspot").

Gene	Type
ASXL1	hotspot
BRAF	hotspot
CBL	hotspot
DNMT3A	full
ETV6	full
EZH2	full
FLT3	hotspot
GATA2	full
JAK3	full
KRAS	hotspot
MAP2K1	hotspot
NF1	full
NRAS	hotspot
PTPN11	hotspot
RAC2	full
RAF1	full
RIT1	full
RRAS	full
RRAS2	hotspot
RUNX1	full
SAMD9	full
SAMD9L	full
SETBP1	hotspot
SH2B3	full
SOS1	full
ZRSR2	full

Supplementary Figure 1: Kaplan Meier survival curves of somatic only (orange) and germline *CBL* (purple) patients.



Additional patient vignettes:

Vignette 4: UPN2951

Patient was first noted to have an enlarged spleen by his pediatrician at 9 months of age. A CBC at that time revealed mild anemia and thrombocytopenia, as well as few circulating myeloid precursors and blasts. At 13 months of age, he developed a mild leukocytosis to 17x10^9/L with monocytosis of 4x10^9/L. The patient was identified to have a germline CBL p.C384R alteration with LOH in his bone marrow. The patient was started on 6-mercaptopurine for failure-to-thrive when he was 18 months and continued on the medication for nearly two years. He subsequently discontinued 6-mercaptopurine due to progressive splenomegaly and severe pruritis. Four subsequent months of ruxolitinib treatment did not improve splenomegaly or the allele frequency of the somatic

component of his CBL mutation. He was then admitted for cytotoxic chemotherapy with fludarabine and cytarabine for two cycles due to massive splenomegaly. The patient had a mild reduction in his splenomegaly and no reduction in the allele burden of his somatic CBL mutation. The patient is remains symptomatic with splenomegaly, pruritus, and episodic weakness and under current evaluation for splenectomy and/or hematopoietic stem cell transplantation.

Vignette 5: UPN2923

A 1 month-old male with no dysmorphic features was found to have splenomegaly on routine examination. A CBC revealed a WBC of 57x10^9/L, a hemoglobin of 8.7g/dL, and platelets of 50x10^9/L. The patient had leukoerythroblastosis with 25% monocytes, metamyelocytes, promyelocytes, myelocytes, and blasts in his circulating blood. He was found to have a heterozygous germline CBL p.C381R mutation, which was homozygous in the bone marrow. All other testing, including cytogenetics and FISH analysis for MDS/AML translocations and monosomies, were negative. The patient was treated with hydroxyurea and cytarabine and was transplanted at 4 months of life using busulfan, cyclophosphamide, and melphalan conditioning using a matched sibling donor who tested negative for the same CBL mutation. The patient had secondary graft failure at two months post-transplant (100% host chimerism), but now has normal bone marrow morphology and is transfusion-independent despite having mild persistent splenomegaly at 2 cm below the left costal margin. This child has recently been noted to have developmental delay.

Vignette 6: UPN3080

This patient was noted to have splenomegaly at 12 months of life first detected at a routine pediatrician visit. The initial CBC was remarkable for an elevated WBC of 60x10^9/L, with a monocytosis of 22x10^9/L. Hemoglobin was low at 9 g/dL, and his platelets were normal. His fetal hemoglobin was normal at 1.9%. Molecular testing for JMML revealed a somatic-only CBL mutation (c.1111T>C) Y371H at 96%. Germline testing using a buccal swab tissue specimen was negative for CBL mutations.

The patient was then treated with single agent azacitidine, which resulted in clinical improvements in his WBC and decreased spleen size, but no response at the molecular level. After 6 cycles of azacitidine, he experienced worsening splenomegaly and was treated with one cycle of fludarabine and cytarabine, but did not have any appreciable benefit. The patient is currently preparing to receive a hematopoeitic stem cell transplant.

Vignette 7: *UPN2921*

A previously healthy 9 month-old girl with no congenital anomalies presented with left hand swelling and bruising and a high fever. Her CBC revealed a WBC of 33x10^9/lL, hemoglobin of 8.4 and platelets of 36x10^9/L. Her monocyte count was 15x10^9/L. Bone marrow examination including BCR/ABL1 testing and cytogenetics/FISH were normal. Sequencing revealed a homozygous CBL p.Y371C mutation in her bone marrow and a heterozygous germline mutation. Her spleen was palpable 3 cm below the left costal margin. She experienced several soft tissue infections that resolved with antibiotics. She was started on 6-mercaptopurine and had improvement in her

splenomegaly and normalization of her platelets. The patient was tapered off of 6-mercaptopurine at 3 years of age, but experienced recurrent thrombocytopenia splenomegaly within two months, and treatment was thus restarted.

Supplementary methods:

Next-generation sequencing of JMML-associated mutations

Genomic DNA samples were sequenced using a custom amplicon-based sequencing approach (Paragon Genomics, Hayward, CA, USA) targeting 26 genes that are known to be recurrently mutated in JMML[1] (Supplementary Table 1). Libraries were assessed using the Bioanalyzer High Sensitivity DNA Analysis kit (Agilent, Santa Clara, CA, USA) and quantified using Qubit (Thermo Fisher Scientific, Waltham, MA, USA). Samples were then sequenced on a MiSeq platform (Illumina, San Diego, CA, USA) at 992X mean coverage. A VAF of 0.05 (=5%) at diagnosis was required for reporting.

Targeted MethylSeq library preparation and sequencing

Genomic DNA (300 ng) was bisulfite converted using the TrueMethyl oxBS Module according to protocol (Tecan, Switzerland). Converted ssDNA was quantified using the Qubit ssDNA assay and 100 ng used as input for a custom 3000 CpG loci targeted MethylSeq assay (Tecan). Final libraries were quality checked using the Bioanalyzer High Sensitivity DNA Analysis kit and quantified using the Qubit HS assay. Single-index library pools were sequenced on the Illumina NovaSeq with paired-end 150 base-pair (bp) mode averaging 20 million reads per library prep.

MethylSeq hierarchical clustering

Sequence reads were trimmed using Cutadapt[2], and DNA methylation status was called using Bismark[3]. CpGs that had ≤50x median coverage were removed from downstream analysis, resulting in 93 CpG sites removed. Minimum distance to the centroid was used to classify samples into one of three DNA methylation subgroups based on a 1386 CpG loci consensus signature that was identified from an international JMML cohort[4].

1. Hecht A, Meyer J, Chehab FF, et al. Molecular assessment of pretransplant chemotherapy in the treatment of juvenile myelomonocytic leukemia. Pediatr Blood Cancer. 2019;66(11):e27948.
2. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. 2011. 2011;17(1):3.
3. Krueger F, Andrews SR. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. Bioinformatics. 2011;27(11):1571-1572.
4. Schönung M, Meyer J, Nöllke P, et al. International consensus definition of DNA methylation subgroups in juvenile myelomonocytic leukemia. Clin Cancer Res. in press;

Supplementary Table 2. Complete clinical data of all patients.

Project ID	UPN	CBL category	Gender	Age at Diagnosis (years)	WBC at Diagnosis (x10^9/l)	Platelets at Diagnosis (x10^9/l)	Hb at Diagnosis (g/dl)
1	2025	CBL syndrome	Male	0.9	38.5	189	9.5
2	1778	CBL syndrome	Male	1.8	19	105	10.9
3	2057	CBL syndrome	Male	2.3			
4	2056	CBL syndrome	Male	2.3			
5	2921	CBL syndrome	Female	0.8	36.35	59	9.1
6	2010	CBL syndrome	Female	1.3	29.2	68	10.3
7	2309	CBL syndrome	Female	1.1	73.1	57	9.9
8	1241	CBL syndrome	Female	0.6	43	62	9.2
9	2903	Somatic only	Female	3.5	33.4	109	11.7
10	2515	CBL syndrome	Female	1.7			
11	1333	CBL syndrome	Male	0.6	35.6	46	8.2
12	1125	CBL syndrome	Female	1.2	196	47	6.2
13	2951	CBL syndrome	Male	1.1	17.5	102	10.3
14	2949	CBL syndrome	Female	25.3			
15	1886	CBL syndrome	Female	0.2	21	204	11.7
16	2178	CBL syndrome	Female	0.8	35	10	6.4
17	2208	CBL syndrome	Female	0.5	21	71	9.9
18	2960	CBL syndrome	Female	2.0	21.96	180	10.5
19	2977	CBL syndrome	Female	0.8	52	59	7.4
20	2760	CBL syndrome	Female	1.5	122	47	9.2

Supplementary						
Project ID	**Hemoglobin F Category**	**Cytogenetics**	**Monosomy 7**	**Splenomegaly**	**Dysmorphic features present**	**Specification of dysmorphic features**
1	Normal	Normal	No	Yes	No	
2	Normal	Normal	No	Yes	Yes	Pectus excavatum, Feet pronation, Some gross motor delay
3				Yes	Yes	Short stature, short neck, low set ears, hat nasal bridge telorism, hypospadia
4		Normal	No	Yes	Yes	Hypertelorism, short stature, webbed neck, low set ears; choanal atresia
5	Not Available	Normal	No	Yes	No	
6	Elevated	Normal	No	Yes	No	
7	Normal	Normal	No	Yes	N/A	
8	Normal	Normal	No	Yes	No	
9	Elevated	Normal	No	Yes	Yes	Examination shows an inner canthal distance of 3.3 centimeters, which is greater than 2 standard deviations above the mean. Bilateral epicanthal folds present with mild eyelid ptosis and mildly up-slanting palpebral fissures. Ears are mildly low-set and posteriorly rotated with a slightly thickened upper helix
10					No	
11	Normal	Abnormal	No	Yes		
12	Not Available	Normal	No	No		
13	Normal	Normal	No	Yes	Yes	Ptosis of right eye Hypotonia Persistent diffusion restriction at bilateral globus pallidi on MRI
14						
15	Normal	Normal	No	Yes	N/A	
16	Normal	Normal	No	Yes	No	
17	Normal	Normal	No	Yes	No	
18	Elevated	Normal	No	Yes	No	
19	Normal	Normal	No	Yes	No	
20	Normal	Normal	No	Yes	Yes	frontal bossing

Supplementary					
Project ID	**Other syndromic features**	**Type of Chemotherapy besides transplant**	**HCT?**	**Type of HCT**	**Type of Conditioning**
1	Optic neuritis/vasculitis, resolved	None	No		
2		None	No		
3	Renal dysplasia/hypoplasia; severe vesicoureteral reflux; hypothyroidism; seizure disorder	None	No		
4	Chronic renal disease due to chronic severe vesicoureteral reflux; hypothyroidism; seizure disorder	None	No		
5		6MP	No		
6		None	Yes	Unrelated Donor	Busulfan,Cyclophosphamide
7		Ara-C,13-CIS Retinoic Acid,Fludarabine	Yes	Unrelated Donor	
8		Other	Yes		Busulfan,Cyclophosphamide,Thiotepa
9		Other	Yes	Cord	Busulfan,Cyclophosphamide,Melphalan
10	Hypothyroidism; bilateral foot eversion	6MP	Yes	Family Donor	Busulfan,Melphalan
11		None	Yes		
12			Yes		
13	Generalized hypotonia, feeding difficulties, failure to thrive; Brachyacephaly	6MP,Ara-C, Fludarabine,Ruxolitinib	No		
14					
15	Usher's syndrome: Retinitis pigmentosa, progressive vision loss; profound sensorineural deafness	None	No		
16		None	Yes	Cord	Busulfan,Melphalan,Fludarabine
17	ASD; short stature	6MP,Ara-C	Yes	Unrelated Donor	Busulfan,Fludarabine,ATG
18		None			
19		6MP,Ara-C, Fludarabine,Azacitidine	Yes	Haploidentical	Busulfan,Cyclophosphamide,Melphalan,Rituximab
20		6MP	No		

Supplementary		
Project ID	**Outcome**	**Inheritance of mutation**
1	Spontaneous remission	De Novo
2	Spontaneous remission	Unknown
3	Persistent Disease, untransplanted; Death from multi-organ complications	De novo
4	Persistent Disease, untransplanted; Death from multi-organ complications	De novo
5	Persistent Disease, untransplanted	Father
6	Remission after transplant	
7	Remission after transplant	Mother
8	Relapse	De novo
9	TRM	N/A
10	Remission after transplant	Unknown
11	Relapse	Mother
12	TRM	Mother
13	Persistent Disease, untransplanted	Father
14	Lost to follow-up	Unknown
15	Spontaneous remission	Unknown
16	Remission after transplant	De Novo
17	Remission after transplant	
18	Persistent Disease, untransplanted	De Novo
19	Remission after transplant	Unknown
20	Persistent Disease, untransplanted	De novo

Supplementary Table 2. Complete clinical data of all patients.

Project ID	UPN	CBL category	Gender	Age at Diagnosis (years)	WBC at Diagnosis (x10^9/l)	Platelets at Diagnosis (x10^9/l)	Hb at Diagnosis (g/dl)
21	2786	CBL syndrome	Male	0.6	21.7	83	
22	2811	Somatic only	Female	1.1	24.1	95	
23	2923	CBL syndrome	Male	0.1	57	50	8.7
24	2985	Somatic only	Female	0.9	81.9	42	10.4
25	3003	CBL syndrome	Female	0.7	80	161	10.3
26	3074	Somatic only	Male	0.6	88		
27	3029	CBL syndrome	Male	2.1	6.85	17	8.7
28	3082	CBL syndrome	Male	0.1	20		
29	3080	Somatic only	Male	1.0	43	167	8.9
30	3102	CBL syndrome	Female	1.7	20	58	10.1
31	3110	CBL syndrome	Male	1.2	34.2	133	9.1
32	2357	CBL syndrome	Male	0.7	19.3	57	11.8
33	2980	CBL syndrome	Female	2.3	18	115	10.7

Supplementary						
Project ID	**Hemoglobin F Category**	**Cytogenetics**	**Monosomy 7**	**Splenomegaly**	**Dysmorphic features present**	**Specification of dysmorphic features**
21	Normal	Normal	No	Yes	Yes	
22	Normal	Normal	No			
23	Normal	Normal	No	Yes	No	
24	Normal	Normal	No	Yes	No	
25	Not Available	Normal	No	Yes	N/A	
26		Normal	No	Yes		
27	Elevated	Normal	No	Yes	No	
28			No	Yes	N/A	
29	Normal	Normal	No	Yes	No	
30	Elevated	Normal	No	Yes	Yes	Mild hypertelorism
31	Elevated	Normal	No	Yes	No	
32	Normal	Normal	No	Yes		
33	Normal	Normal	No	Yes	No	

Supplementary					
Project ID	**Other syndromic features**	**Type of Chemotherapy besides transplant**	**HCT?**	**Type of HCT**	**Type of Conditioning**
21		6MP	Yes	Haploidentical	Busulfan,Cyclophosphamide,post-transplant Cy
22		6MP,Ara-C,Fludarabine	Yes	Family Donor	Busulfan,Cyclophosphamide,Melphalan
23		Ara-C,HU	Yes	Family Donor	Busulfan,Cyclophosphamide,Melphalan
24		Ara-C,Fludarabine	Yes	Family Donor	Busulfan,Cyclophosphamide,Melphalan
25	Asthma	6MP			
26		Ara-C,Fludarabine,Trametinib	No		
27		6MP,Sirolimus	No		
28	Absence of thoracic duct, pulmonary stenosis, intestinal mal rotation, liver dysfunction, chronic respiratory failure, hydronephrosis, congenital nystagmus and esotropia	None	No		
29		Ara-C, Fludarabine,Azacitidine,Trametinib	No		
30		None	No		
31		6MP	No		
32			Yes	Cord	
33		None	No		

Supplementary		
Project ID	**Outcome**	**Inheritance of mutation**
21	Remission after transplant	Mother
22	Remission after transplant	N/A
23	Remission after transplant	Unknown
24	Remission after transplant	N/A
25	Persistent Disease, untransplanted	Unknown
26	Persistent Disease, untransplanted	N/A
27	Persistent Disease, untransplanted	Father
28	Multi-organ failure in infancy	Unknown
29	Persistent Disease, untransplanted	N/A
30	Persistent Disease, untransplanted	Father
31	Persistent Disease, untransplanted	
32	Remission after transplant	Unknown
33	Persistent Disease, untransplanted	Unknown

Supplementary Table 3. Mutational data and methylation status of all patients.

Project ID	UPN	CBL Aberration	Tumor VAF %	Germline heterozygous	Methylation group	Additional comments
1	2025	p.Y371H	90	positive	low	
2	1778	p.Y371N	90	positive	low	
3	2057	p.Q358fs	69	positive	low	
4	2056	p.Q358fs	81	positive	low	
5	2921	p.Y371C	92	positive	low	
6	2010	p.C396R	97	positive	low	
7	2309	p.Y371H	94	positive	low	
8	1241	p.Y371H	90	positive	low	
9	2903	p.Y371H	72	negative	intermediate	heterozygous RUNX1 p.R166Q found in germline
10	2515	del21bp intron 8-exon 8	75	positive	low	
11	1333	p.Y371H	76	positive	intermediate	
12	1125	p.Y371H	95	positive	low	
13	2951	p.C384R	89	positive	low	
14	2949	p.C396R	99	positive	low	
15	1886	exon 8 del	1 copy loss	positive	low	
16	2178	p.Y371H	97	positive	low	
17	2208	p.C384G	97	positive	low	
18	2960	p.C381Y	84	positive	low	
19	2977	p.Y371H	91	positive	low	
20	2760	p.Y371H	97	positive	low	
21	2786	c.1228-2 A>G	95	positive	low	
22	2811	p.C404R	97	negative	low	
23	2923	p.C381R	97	positive	low	
24	2985	p.C381R	93	negative	low	
25	3003	p.C381R	95	positive	low	
26	3074	p.Y371H	45	negative	low	
27	3029	p.C419F	90	positive	low	
28	3082	c.1098-1 G>T	51	positive	low	
29	3080	p.Y371H	96	negative	low	
30	3102	p.C284R p.Y371H	64 33	positive	low	

Supplementary Table 3. Mutational data and methylation status of all patients.						
Project ID	**UPN**	**CBL Aberration**	**Tumor VAF %**	**Germline heterozygous**	**Methylation group**	**Additional comments**
31	3110	p.R420P	90	positive	low	
32	2357	c.1228-2 A>G	91	positive	low	
33	2980	p.M400R	100	positive	low	