LNK/*SH2B3* as a novel driver in juvenile myelomonocytic leukemia

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Abstract

Mutations in five canonical Ras pathway genes (*NF1, NRAS, KRAS, PTPN11* and *CBL*) are detected in nearly 90% of patients with juvenile myelomonocytic leukemia (JMML), a frequently fatal malignant neoplasm of early childhood. In this report, we describe seven patients diagnosed with *SH2B3*-mutated JMML, including five patients who were found to have initiating, loss-of-function mutations in the gene. *SH2B3* encodes the adaptor protein LNK, a negative regulator of normal hematopoiesis upstream of the Ras pathway. These mutations were identified to be germline, somatic or a combination of both. Loss of function of LNK, which has been observed in other myeloid malignancies, results in abnormal proliferation of hematopoietic cells due to cytokine hypersensitivity and activation of the JAK/STAT signaling pathway. *In vitro* studies of induced pluripotent stem cell-derived JMML-like hematopoietic progenitor cells also demonstrated sensitivity of *SH2B3*-mutated hematopoietic progenitor cells to JAK inhibition. Lastly, we describe two patients with JMML and *SH2B3* mutations who were treated with the JAK1/2 inhibitor ruxolitinib. This report expands the spectrum of initiating mutations in JMML and raises the possibility of targeting the JAK/STAT pathway in patients with *SH2B3* mutations.

Introduction

Juvenile myelomonocytic leukemia (JMML) is a rare and aggressive overlapping myelodysplastic/myeloproliferative disorder in toddlers with a median age at onset of approximately 2 years.[1] Outcomes range from spontaneous remission in some patients to aggressive disease and transformation to acute myeloid leukemia in others. Most patients undergo hematopoietic cell transplantation (HCT) with curative intent. At diagnosis, a high white blood cell count with circulating immature myeloid cells, a peripheral monocytosis, nucleated red blood cells, thrombocytopenia, elevated fetal hemoglobin, and splenomegaly are typically observed. Fever, cough, bloody stools, and failure to thrive may also be present. Bone marrow aspirates must display fewer than 20% blasts and can have varying degrees of abnormal erythro-, myelo- and megakaryo-poiesis. Historically, laboratory features including hypersensitivity of myeloid progenitor cells to granulocyte-macrophage colony-stimulating factor (GM-CSF) in colony-forming assays or hyperphosphorylation of STAT5 of CD38-positive cells were used to establish a diagnosis of JMML.[2] Currently, next-generation sequencing is considered standard-of-care and allows for an accurate diagnosis as nearly all patients with JMML (~95%) have mutations detected in the Ras/MAPK signaling pathway genes including *CBL, KRAS, NF1, NRAS, RRAS, RRAS2*, and *PTPN11*.[3-6] The vast majority of these driver mutations are mutually exclusive and can be acquired in a germline and/or somatic configuration. One consequence of these mutations is hyperactivation of the Ras/MAPK pathway, including Raf/MEK/ERK. Secondary mutations at a lower allele frequency are often found outside the canonical Ras pathway and include alterations in transcription factors, epigenetic regulating genes, and the spliceosome complex. These additional mutations contribute to disease progression and predict poor outcome.[3,5] In addition to the commonly mutated genes listed above, oncogenic fusion proteins that lead to hyperactive Ras signaling,[7-10] as well as mutations in other genes encoding for proteins upstream of the Ras pathway (e.g. *FLT3*) have been described in rare patients.[10,11] One of these upstream proteins is the lymphocyte adaptor protein LNK that is encoded by the *SH2B3* gene on chromosome 12q24.12. We previously identified seven patients with secondary mutations in *SH2B3* in a genomic characterization of 100 patients with JMML.[3] Herein, we report seven new patients, including five with initial mutations in *SH2B3* and two with secondary *SH2B3* mutations. We also show that *SH2B3*-mutated induced pluripotent stem cell (iPSC)-derived JMML-like hematopoietic progenitor cells (HPC) are sensitive to JAK inhibitors, including ruxolitinib and momelotinib. Importantly, we describe two patients with *SH2B3*-mutated JMML treated with ruxolitinib who experienced clinical responses, highlighting the potential relevance of this precision medicine approach in JMML.

Methods

Primary patients' samples

The patients' guardians provided informed consent to this study which was reviewed and approved by the institutional review board of the University of California San Francisco (institutional review board number: 10-0421) in accordance with the Declaration of Helsinki. Genomic DNA from peripheral blood, bone marrow or buccal swabs was extracted using standard protocols. DNA samples were sequenced using a custom amplicon-based targeted sequencing approach. Methylation profiles were analyzed according to previously published protocols[12] and annotated according to the international, consensus definition.[13] Additional details are described in the *Online Supplementary Methods.*

Generation of induced pluripotent stem cells

Primary JMML and control samples were obtained at Benioff Children's Hospital at the University of California San Francisco or received from other pediatric institutions via a locally-approved institutional review board research protocol. Ficoll-purified mononuclear cells from bone marrow were reprogrammed by using the Sendai virus expressing doxycycline-regulated OCT4, KLF4, MYC, and SOX2 as previously described at the Children's Hospital of Philadelphia.[14] All iPSC studied fulfilled standard pluripotency criteria, including expression of endogenous pluripotency markers, silencing of Sendai virally-encoded reprogramming genes, and formation of all three germ-cell layers. A list of iPSC generated for this study can be found in *Online Supplementary Table S1.*

Differentiation of induced pluripotent stem cells to hematopoietic progenitor cells

Control and JMML iPSC were differentiated by culturing cells in serum-free media with sequential combinations of cytokines (all growth factor reagents from R&D Systems) to support multipotent hematopoietic progenitor formation as previously described.[15] Additional details are described in the *Online Supplementary Methods.*

Cell viability assay

The half-maximal inhibitory concentration (IC_{50}) for each kinase inhibitor was determined by performing luminescence-based Cell Titer Glo assays (Promega) according to the manufacturer's protocol with readout at 72 hours (h). Each agent (ruxolitinib, momelotinib, tofacitinib) was tested at three different times with each concentration tested in triplicate.

Induced pluripotent stem cell-derived hematopoietic progenitor cell drug discovery screen

A small molecule discovery screen was performed, in collaboration with the University of California San Francisco Small Molecule Discovery Center, in HPC collected on day 10 of monolayer differentiation from iPSC carrying mutations.

Five thousand HPC were plated into each well of a 384-well assay plate in 50 µL of HPC-propagating media and treated with the compound library of approximately 2,000 bioactive substances at 125 nM for 72 h in triplicate. The effect on viability was measured using Cell Titer Glo assays as above. Percent inhibition was calculated relative to positive and negative controls with the negative control equivalent to 0% inhibition (no compound added) and the positive control equivalent to 100% inhibition (no cells added). Percent inhibition of each mutant line was then compared to the percent inhibition of the wild-type/non-mutant (WT) control. Additionally, hits against single-mutant HPC were compared with hits against double-mutant HPC. Statistical analyses and graphic data display were performed with R (version 3.6).

Single-cell DNA and protein sample preparation, sequencing, and data analysis

Unsorted mononuclear cells from patient UPN2861 at the time of diagnosis were analyzed using a single-cell microfluidic approach with molecular barcode technology. Details of this approach, including generation of the phylogenetic tree, are described in the *Online Supplementary Methods* (including *Online Supplementary Table S5*).

Results

SH2B3 mutations frequently co-occur with *PTPN11*

In patients who met criteria for JMML we identified germline and/or somatic mutations in *SH2B3* that resulted in a truncated LNK protein or affected the biologically important SH2 domain (Figure 1, *Online Supplementary Figure S2*). Molecular and clinical characteristics of the seven patients reported for the first time are summarized in Tables 1 and 2, respectively. Including previously reported cases,[3] seven of 14 patients with *SH2B3*-mutated JMML also harbored somatic *PTPN11* mutations.

Induced pluripotent stem cell-derived hematopoietic progenitor cells recapitulate juvenile myelomonocytic leukemia

To investigate the cooperative nature of *SH2B3* and *PTPN11* mutations, we generated iPSC-derived HPC with one or both mutations. To confirm that HPC recapitulate JMML, we performed colony-formation assays at increasing doses of GM-CSF. While WT HPC formed almost no colonies in the absence of GM-CSF, *PTPN11*-mutant and *PTPN11/SH2B3*-mutant HPC formed significantly more colonies (P=0.0004 for WT *vs.* *PTPN11* and P<0.0001 for WT *vs.* *PTPN11/SH2B3*) (*Online Supplementary Figure S3A*). Mutant HPC derived from iPSC showed spontaneous proliferation independent of GM-CSF, an important hallmark of JMML. Elevated signaling of STAT5 and ERK, another characteristic of JMML cells, was also observed in HPC, more prominently in the *PTPN11/SH2B3* double-mutant HPC (*Online Supplementary Figure S3B*).

Drug discovery screen identified JAK inhibitors with differential effects on cell proliferation depending on mutational background

In an independent high-throughput drug discovery screen performed using single- and double-mutant iPSC-derived JMML-like HPC, we identified multiple JAK1/2 inhibitors

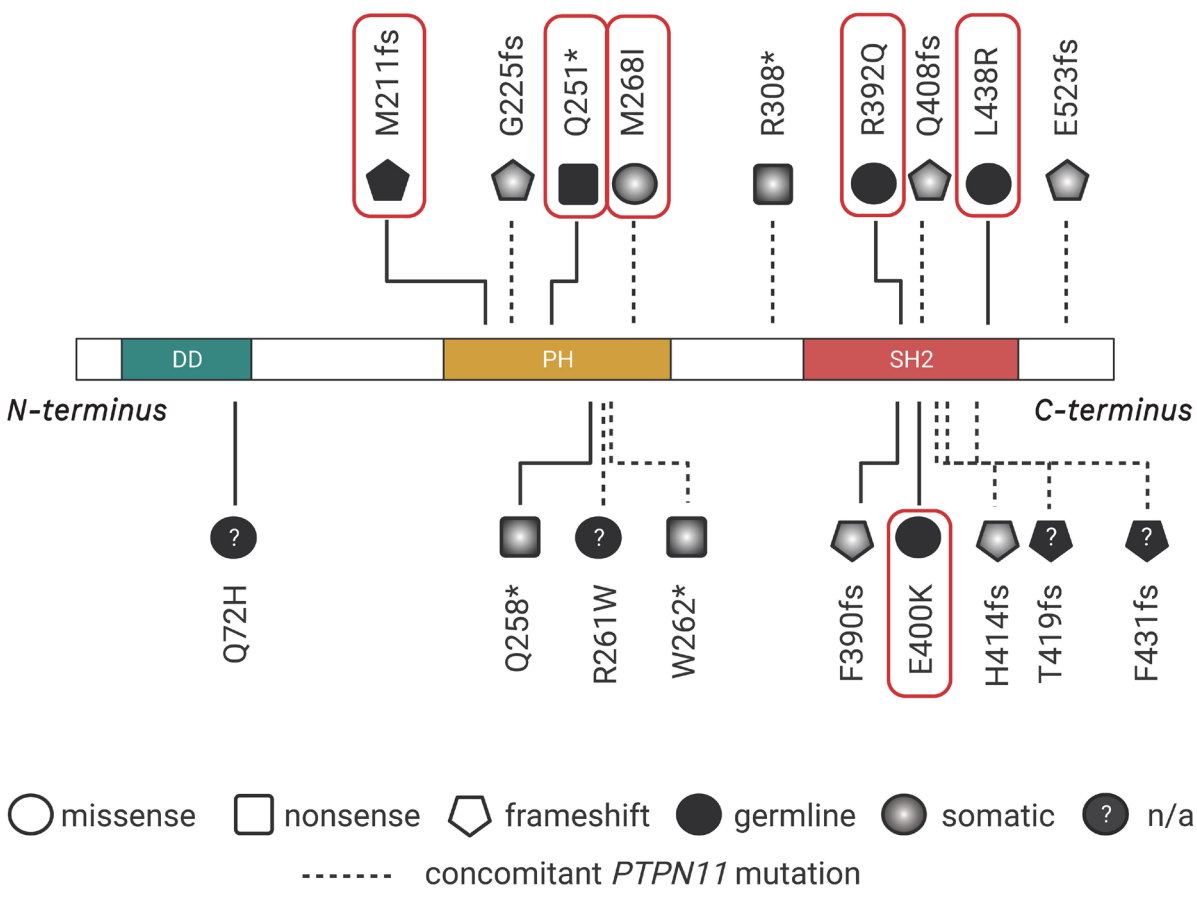


Figure 1. Schematic overview of *SH2B3* including the location of both primary and secondary mutations described in juvenile myelomonocytic leukemia. The top row shows the mutations of the seven novel patients reported here; the bottom row shows the location of the mutations previously reported by our group.[3] Mutations that are considered to initiate juvenile myelomonocytic leukemia are highlighted in red boxes. Alterations that co-exist with a *PTPN11* mutation are displayed with a dashed line. DD: dimerization domain; PH: pleckstrin homology domain; SH2; Src homology 2 domain; n/a: not available.

among the top ten compounds that showed a greater inhibition of *PTPN11/SH2B3*-mutant HPC compared to *PTPN11*-mutant HPC (Figure 2A, *Online Supplementary Table S2*).

SH2B3-mutant hematopoietic progenitor cells are more sensitive to JAK inhibitor therapy

To validate the drug discovery screen, we analyzed cell proliferation of iPSC-derived HPC with different mutational backgrounds after exposure to various JAK inhibitors, including ruxolitinib, momelotinib, and tofacitinib. HPC with alterations in *SH2B3* were more sensitive to chemical JAK inhibition compared to HPC not harboring mutations in *SH2B3*. This finding was observed for all JAK inhibitors but was most striking for ruxolitinib (Figure 2B).

Table 1. Molecular characteristics of the seven patients with *SH2B3* mutations.

Characteristics	UPN2861	UPN3426	UPN3436	UPN1744	UPN3037	UPN3160	UPN2823
Sex	M	M	F	F	F	M	M
Age at diagnosis	4 years	0 months	4 months	2 months	5 years	4 months	6 years
SH2B3 - primary or secondary	Primary	Primary	Primary	Primary	Secondary	Primary	Unknown
SH2B3 alteration (VAF%)	p.M268I (86)	p.L438R (100)	p.R392Q (100)	p.Q251* (63)	p.Q408fs (38); p.E523fs (18)	p.M211fs*57 (100)	p.R308* (46); p.G225fs*47 (21)
Configuration of *SH2B3* alteration	Somatic	Germline	Germline	Germline	Somatic	Germline	Somatic
Other additional alterations (VAF%)	*PTPN11* p.A72T (83); *WT1* p.K492Q (12); *IKZF1* p.F154Y (8)	None	None	*NF1* p.Y628fs (5)	*PTPN11* p.E76V (46)	None	*RRAS* p.Q72L (40); *ZRSR2* p.Q255 (19); *PTPN11* p.T73I (4)
Cytogenetic abnormalities	No	No	No	No	No	No	No
Methylation profile	High	Low	NA	Low	High	Low	High

UPN: unique patient number; M: male; F: female; VAF: variant allele frequency; NA: not available.

Table 2. Clinical characteristics of the seven patients with *SH2B3* mutations.

Characteristics	UPN2861	UPN3426	UPN3436	UPN1744	UPN3037	UPN3160	UPN2823
Hb at diagnosis, g/dL	11.1	11.2	10	9.9	10.7	9.8	10.2
WBC at diagnosis, $x10^9$/L	501	69.7	102	84.9	67	114	11.4
Platelets at diagnosis, $x10^9$/L	13	49	75	50	102	181	116
Monocytes at diagnosis, $x10^9$/L	>8	3.1	13	Unknown	7.14	10	1.63
HbF at diagnosis	Elevated	Not done	Elevated	Not done	Elevated	Normal	Elevated
Peripheral blast count at diagnosis, %	6	9	5	Unknown	6	5	12
Splenomegaly at diagnosis	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Circulating myeloid or erythroid precursors	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Treatment	HCT	Chemotherapy	HCT	HCT	HCT	HCT	HCT
Ruxolitinib	No	No	No	No	Yes	Yes	No
Outcome	Died	Alive	Alive	Alive	Died	Alive	Alive

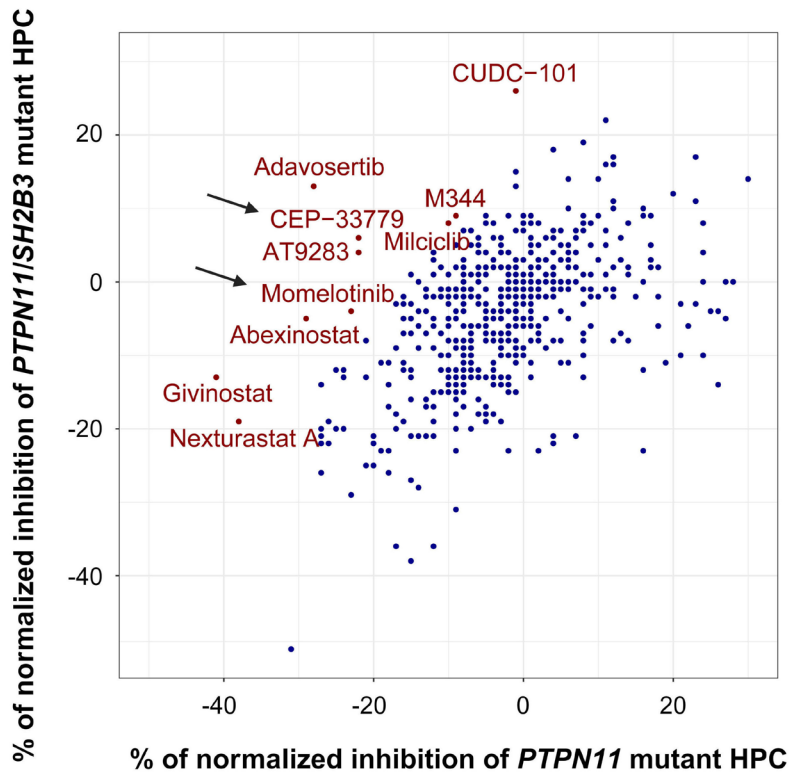
UPN: unique patient number; Hb: hemoglobin; WBC: white blood cells; HbF: fetal hemoglobin; HCT: hematopoietic stem cell transplantation.

Single-cell sequencing revealed the phylogenetic origin in a patient with concomitant *SH2B3* and *PTPN11* mutations

We identified a patient with a *PTPN11* p.A72T mutation at an unusually high variant allele frequency (VAF) (83%) along with a *SH2B3* p.M268I mutation (VAF 86%). This previously healthy 4-year-old male (UPN2861) was diagnosed with JMML after presenting with petechiae and splenomegaly and a complete blood count showing leukocytosis (white blood cell count 501x10^9/L), severe thrombocytopenia (platelet count 13x10^9/L), and monocytosis (absolute monocyte count >8x10^9/L). Fetal hemoglobin was elevated at 65% and cytogenetic and fluorescence *in situ* hybridization (FISH) analyses were normal. To determine the sequence of mutational acquisition, single-cell sequencing was performed, which revealed that a somatic *SH2B3* p.M268I was the initial mutation, and then branched into a *PTPN11* p.A72T population and a homozygous *SH2B3* p.M268I population (Figure 3, *Online Supplementary Table S3*).

Homozygous or heterozygous *SH2B3* mutations in the germline can lead to juvenile myelomonocytic leukemia

Recognizing that mutations in *SH2B3* can initiate JMML, we screened additional JMML patients without any known driver mutation. A male (UPN3426) with consanguineous parents was born at a gestational age of 33 weeks by Cesarean section because of intrauterine growth retardation and was found to have intracranial and intrahepatic calcifications, hepatosplenomegaly, and thrombocytopenia as well as leukocytosis with monocytosis. An extensive infectious disease workup was negative. Bone marrow examination (*Online Supplementary Figure S1C, D*) revealed 9% myeloblasts and cytogenetic analysis demonstrated a normal male karyotype. A diagnosis of JMML was established. The patient developed progressive splenomegaly, portal hypertension and transfusion dependency and was started on low-dose



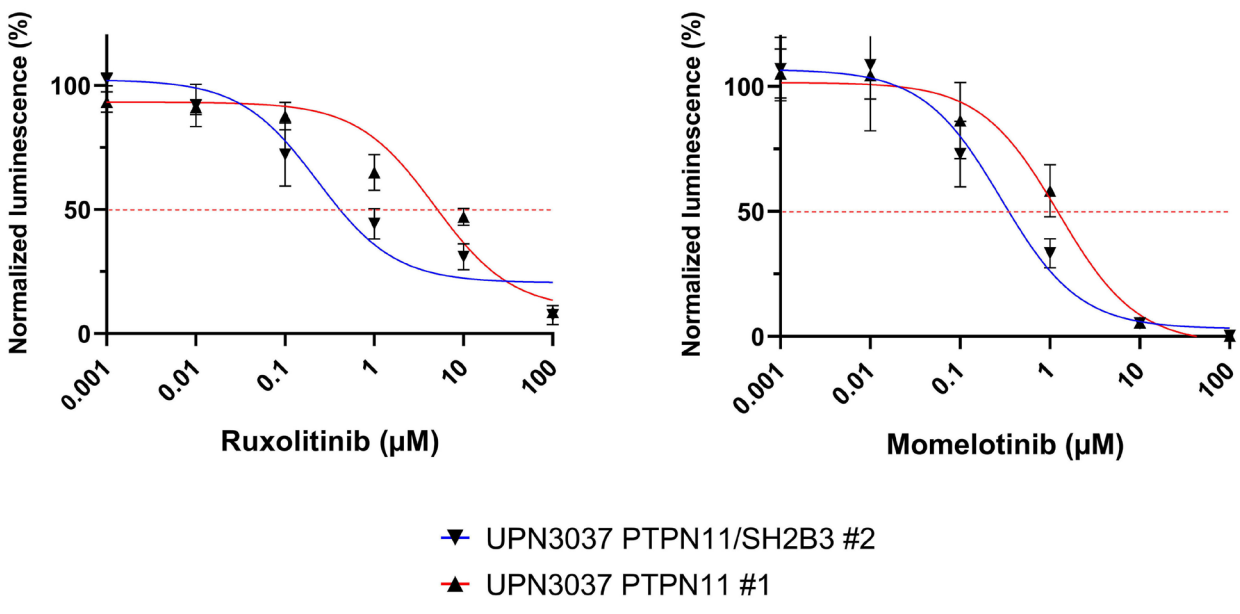


Figure 2. *SH2B3*-mutated hematopoietic progenitor cells are more sensitive to JAK inhibitor therapy. (A) Linear regression plot of a high throughput drug discovery screen comparing drug inhibition of *PTPN11/SH2B3* double-mutant hematopoietic progenitor cells (HPC) *versus PTPN11* single-mutant HPC. The top ten hits that inhibited growth of double-mutant HPC to a greater extent than that of single-mutant HPC include two JAK inhibitors: momelotinib and CEP-33779. (B) Cell viability assay readout 72 hours after exposing two different induced pluripotent stem cell-derived HPC lines to ruxolitinib or momelotinib. Data for tofacitinib are not shown. HPC: hematopoietic progenitor cells.

cytarabine and 6-mercaptopurine. Symptoms improved and both medications were eventually discontinued by 20 months of life. The patient has since developed thrombocytosis (platelet counts 800-1,200x10^9/L) and continues to have splenomegaly but is otherwise asymptomatic and thriving. Next-generation sequencing identified a germline *SH2B3* p.L438R mutation (VAF 100%) in the patient and both parents were found to be heterozygous germline carriers of the same mutation.

A female (UPN3436) with consanguineous parents was born at term via Cesarean section and was found to have low birth weight and hepatosplenomegaly. She was admitted because of neonatal jaundice. At the age of 4 months, she presented with recurrent fever and diarrhea. A complete blood count demonstrated leukocytosis, anemia and thrombocytopenia. An extensive infectious and metabolic disease workup was negative. Bone marrow examination revealed dysmegakaryopoiesis with 4% blasts (*Online Supplementary Figure S1E, F*). A diagnosis of JMML was established and the patient underwent HCT. Next-generation sequencing of a peripheral blood sample revealed a *SH2B3* p.R392Q mutation (VAF 100%). Sanger sequencing of a buccal swab demonstrated the same homozygous *SH2B3* mutation. Parental DNA was not available for testing.

A 2-month-old female (UPN1744) presented with leukocytosis, thrombocytopenia and splenomegaly. A peripheral blood smear demonstrated circulating myeloid precursor cells and a bone marrow aspirate was consistent with JMML. She was treated briefly with low-dose cytarabine before receiving a 4/6 human leukocyte antigen-matched unrelated cord blood transplant after conditioning with busulfan, cyclophosphamide, melphalan and anti-thymocyte globulin. The patient developed chronic graft-*versus*-host disease of the skin but is currently alive and well with no signs of disease 14 years after the transplant. Next-generation sequencing of the peripheral blood identified a *SH2B3* p.Q251* mutation (VAF 63%). Sanger sequencing of T cells confirmed the same heterozygous mutation in the germline. Parental DNA was not available for testing.

Ruxolitinib led to resolution of splenomegaly in a patient with secondary *SH2B3* mutations

A previously healthy, 5-year-old female (UPN3037) presented with fever, leukocytosis, monocytosis, thrombocytopenia and splenomegaly. Fetal hemoglobin was elevated at 63.3% and bone marrow examination showed 6% atypical myeloid blasts. Cytogenetic and FISH analyses were normal. DNA sequencing detected a primary mutation in *PTPN11* p.E76V (VAF 46%) and two secondary *SH2B3* mutations including p.Q408fs (VAF 38%) and p.E523fs (VAF 18%). The diagnosis of JMML was established and the patient was started on ruxolitinib treatment at a dose of 50 mg/m^2 by mouth twice a day. Ten days into ruxolitinib monotherapy, the patient's white blood cell count and monocytosis had decreased and abdominal ultrasound showed resolution of splenomegaly. Bone marrow examination following 10 days of ruxolitinib monotherapy revealed that the VAF of the *SH2B3* mutation at p.Q408fs had decreased to 22%, while the *SH2B3* mutation at p.E523fs was no longer detectable. However, the *PTPN11* p.E76V mutation was unchanged, and a new *NRAS* p.G12D mutation was detected at a VAF of 4% (Figure 4). Fludarabine 30 mg/m^2 daily for 5 days and cytarabine 2 g/m^2 daily for 5 days were added to ruxolitinib, but the patient experienced progressive disease. The patient was then treated sequentially with trametinib and azacitidine but progressed after each treatment. The girl received a haploidentical HCT from her mother following a condition-

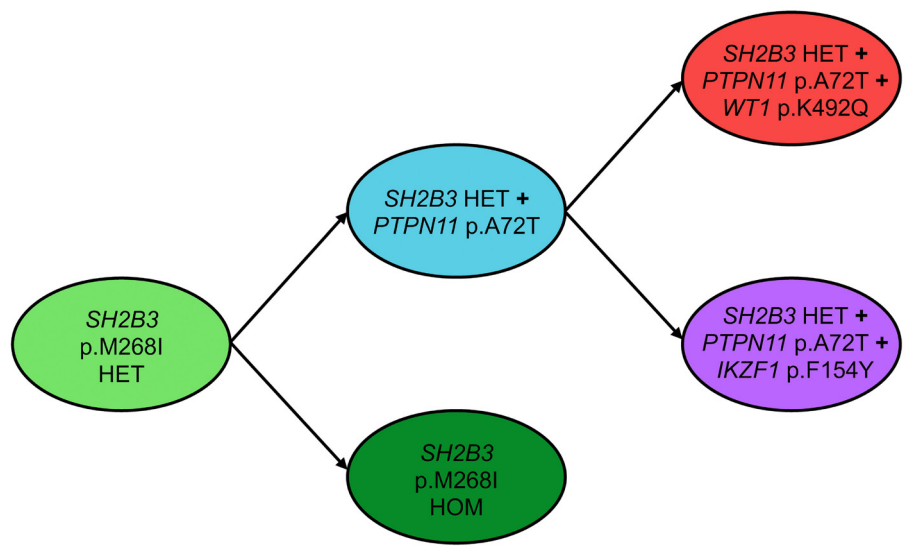


Figure 3. Phylogenetic tree in a patient with juvenile myelomonocytic leukemia. The phylogenetic tree at diagnosis in patient UPN2861 was inferred from single-cell sequencing and single-cell inference of tumor evolution (SCITE), a probabilistic model using a flexible Markov-chain Monte Carlo algorithm.[37] A heterozygous *SH2B3* p.M268I was the initiating mutation, which then branched into a *PTPN11* population and a homozygous *SH2B3* population. The *PTPN11* population finally branched into *WT1* and *IKZF1* clones. HET: heterozygous; HOM: homozygous.

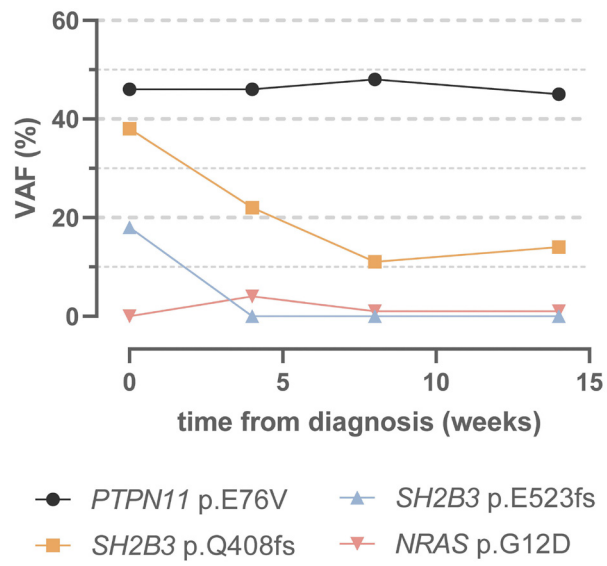


Figure 4. Molecular response of patient UPN3037 who harbored a *PTPN11* and two *SH2B3* mutations at diagnosis. Following 10 days of ruxolitinib monotherapy, the *SH2B3* mutation at codon 523 was no longer detectable, and the allele frequency of the *SH2B3* mutation at codon 408 decreased from 38% to 11%. VAF: variant allele frequency.

ing regimen with busulfan, cyclophosphamide, thiotepa, anti-thymocyte globulin and total body irradiation. She relapsed by day +90 and subsequently received a paternal haploidentical HCT. She developed idiopathic pulmonary syndrome and died of respiratory failure in a molecular remission from JMML at day +60.

Ruxolitinib as a bridge to hematopoietic stem cell transplantation in a patient with *SH2B3*-mutated juvenile myelomonocytic leukemia

A 4-month-old male (UPN3160) was diagnosed with JMML after presenting with anemia, leukocytosis with peripheral monocytosis, 5% circulating myeloblasts, and hepatosplenomegaly. A bone marrow biopsy revealed myeloid hyperplasia (*Online Supplementary Figure S1A*) and cytogenetic and FISH analyses were normal. DNA sequencing revealed a *SH2B3* p. M211fs*57 mutation at 50% VAF in the germline and 100% VAF in the tumor due to copy neutral loss of heterozygosity from 12q21.1 to 12q24.33. The germline mutation was discovered to be maternally inherited. A diagnosis of JMML was made and the patient was started on 6-mercaptopurine, but splenomegaly persisted. The patient was then started on ruxolitinib monotherapy at a dose of 15 mg/m^2 by mouth twice daily which led to complete resolution of splenomegaly, but no change in the VAF of the *SH2B3* mutation, which remained at 100%. The patient continued on single-agent ruxolitinib as a bridge to HCT and is now in a molecular remission 2 years after the transplant.

Discussion

LNK is a member of the SH2-B family of adaptor proteins that share three functional domains: a dimerization domain at the N terminus, a central pleckstrin homology (PH) domain and a C-terminal Src homology 2 (SH2) domain. LNK is mainly expressed in hematopoietic cells, particularly in hematopoietic stem cells.[16] Most of the protein remains in the cytoplasm, specifically in the perinuclear region.[17,18] However, the PH domain allows for binding to the plasma membrane via interaction with membrane phospholipids. The SH2 domain is responsible for most of the biological effects of LNK through interaction with phosphorylated signaling partners including cytokine and tyrosine kinase receptors (EPO, TPO, SCF) and kinases (JAK2).[19,20]

The generation of LNK-deficient mice elucidated the role of LNK in hematopoiesis: *Lnk*$^{-/-}$ mice developed features of myeloproliferative disease including splenomegaly, increased numbers of myeloid progenitors and extramedullary hematopoiesis.[16,21] A significant accumulation of pro- and pre-B cells was also noted in *Lnk*$^{-/-}$ mice, demonstrating a role of LNK as a negative regulator in B-lymphopoiesis.[22] These findings are thought to be caused (at least in part) by the hypersensitivity of *Lnk*$^{-/-}$ progenitors to several cytokines, with increased activation of STAT3, STAT5, AKT and MAPK signaling pathways.[23]

It is therefore not surprising that mutations in *SH2B3* have been identified in a variety of hematologic malignancies.[24] Mutations in *SH2B3* have been reported in 5-7% of patients with myeloproliferative neoplasms across all subtypes[25-27] and increase up to 13% upon leukemic transformation.[28] *SH2B3* mutations have also been described in lymphoid malignancies, albeit at a much lower frequency.[27,29] In a previous study of 100 patients with JMML, we identified the first seven patients with *SH2B3* mutations.[3] While six of the previously reported patients harbored secondary *SH2B3* mutations in addition to known JMML driver mutations such as *NF1* or *PTPN11*, one patient had a germline heterozygous *SH2B3* mutation without additional somatic mutations (*Online Supplementary Table S4*). Here, we present five patients with initial mutations and two patients with secondary mutations in *SH2B3* (Table 1). Due to the absence of other disease-driving alterations in patients UPN3426, UPN3436, and UPN3160 as well as a lower allele frequency for the *NF1* mutation in UPN1744 (Table 1), we presume that *SH2B3* mutations initiated JMML in these four patients. Phylogenetic analysis of a sample from UPN2861 using single-cell DNA sequencing determined that the initiating mutation was in *SH2B3*, which then branched into discrete subclones, one of which acquired a secondary *PTPN11* mutation. Methylation profiling showed a low methylation signature for patients UPN3426, UPN3436, and UPN3160 harboring a germline *SH2B3* mutation. Patients UPN2861, UPN3037 and UPN2823, who had multiple mutations present at diagnosis, were categorized as having high methylation signatures. These data are consistent with previous reports that altered methylation frequently accompanies the presence of secondary mutations.[5,13,30]

Several groups have functionally validated *SH2B3* mutations and demonstrated that point mutations in the PH domain impair translocation to the plasma membrane and thus reduce its regulatory function,[18] while mutations in the SH2 domain affect the interaction with JAK/STAT and result in a more severe phenotype.[19,20] The mutations identified here result in a truncated protein (patients UPN3160, UPN2823 and UPN1744) or affect the biologically important SH2 domain (patient UPN3426) (Figure 1). Interestingly, copy-neutral loss of heterozygosity of *SH2B3* in patient UPN3160 associated with uniparental isodisomy is a mechanism that has been observed commonly in other cancers and specifically in JMML with *CBL* and *NF1* mutations.[31,32]

In general, there is remarkable similarity between *SH2B3*-mutated JMML and *CBL*-mutated JMML. Both are associated with germline mutations (including heterozygous germline mutations without any somatic events), can occur in the context of a constitutional syndrome, can lead to upregulation of the JAK-STAT pathway, can be associated with copy-neutral loss of heterozygosity in the tumor, and is often manifested by a spontaneously remitting form of JMML. *SH2B3*-mutated JMML also shares similarities with

myeloproliferative disorders seen in infants with Noonan syndrome, most commonly caused by germline mutations in *PTPN11*. Both can present in the context of a constitutional syndrome and can manifest with a transient myeloproliferative disorder of infancy. Although limited by very small numbers, the severity of the myeloproliferation in our cohort appeared to differ based on whether the *SH2B3* mutations were germline or somatic and whether the former were monoallelic or biallelic. In general, germline mutations were associated with less aggressive disease compared to somatic mutations. Larger studies will be required to validate these initial findings and to determine their exact classification as a myeloproliferative disorder, myeloproliferative neoplasm/myelodysplastic syndrome or JMML.

A schematic overview of all *SH2B3* mutations identified in JMML to date is highlighted in Figure 1. We observed a striking association between *SH2B3* and *PTPN11* with seven of 14 patients harboring both mutations (*Online Supplementary Figure S4*). Of note, *SH2B3* and *PTPN11* are located in close proximity at 12q24.12 and 12q24.13, respectively. We observed copy neutral loss of heterozygosity causing elevated VAF in *SH2B3* and *PTPN11* above what is typically observed in cases with *PTPN11* mutations alone. To model the cooperative nature of these mutations, we engineered iPSC-derived HPC with one or both mutations and observed increased pSTAT5 and pERK signaling in the cells with both mutations compared to one alone. Since *in vitro* data showed that loss of LNK results in increased JAK/STAT signaling, we hypothesize that this cohort of patients may benefit from JAK inhibitor therapy. Our data from iPSC-derived JMML-like HPC show that those cells with secondary *SH2B3* mutations are more sensitive to JAK inhibitors, including ruxolitinib and momelotinib, which are approved by the Food and Drug Administration and European Medicine Agency or under clinical investigation in adults with myeloproliferative neoplasms.[33-35] It is important to note that our iPSC data highlight the efficacy of ruxolitinib in *SH2B3*-mutated JMML but cannot provide insight into the potential relevance of the sequence to acquisition of each mutation. Our findings are consistent with those of a previous study in iPSC which also demonstrated that JAK inhibitor therapy could be beneficial in *CBL*-mutated JMML.[36] Following a 10-day course of treatment with ruxolitinib, patient UPN3037, who harbored a *PTPN11* and two *SH2B3* alterations at diagnosis, had a decrease in white blood cell count and improved splenomegaly. Importantly, the *SH2B3* p.E523fs mutation was no longer detectable and the *SH2B3* p.Q408fs allele frequency was reduced from 38% to 11% (Figure 4) while the patient was receiving ruxolitinib monotherapy. However, ruxolitinib did not have any appreciable effect on the initiating *PTPN11* mutation and the patient experienced progressive disease. Additionally, patient UPN3160 experienced a rapid resolution of splenomegaly after one cycle of ruxolitinib monotherapy and this treatment served as a bridge to HCT.

We have previously reported on a JMML patient with a heterozygous germline *SH2B3* mutation.[3] Here, we have shown that heterozygous germline *SH2B3* mutations can become homozygous in hematopoietic cells due to copy-neutral loss of heterozygosity and that homozygous germline *SH2B3* mutations can all converge on causing JMML. Lastly, we identified a patient with *PTPN11* and *SH2B3*-mutated JMML who, we have now shown using-single cell sequencing, had an initiating somatic mutation in *SH2B3*.

In summary, this report expands the spectrum of driver mutations in JMML that lead to MAPK activation to include *SH2B3*, and highlights JAK/STAT inhibition as a possible targeted treatment for these patients.

Disclosures

No conflicts of interest to disclose.

Contributions

AW performed experiments, analyzed data, and wrote the manuscript. AH performed experiments, analyzed data, and edited the manuscript. JM performed experiments and the bioinformatic analysis. FC helped with the bioinformatic analysis; EBW, JH, SA, and KF performed experiments. VEK, CACP, DLF, CJ, and JAM performed experiments and analyzed data. JM, JH, MRV, SD, RD, EN, FFC, SKT, HH, MH, AEH, SCK, and CCS analyzed data. MLL and ES designed and supervised the project and edited the manuscript. All authors contributed to and approved the final version of the manuscript.

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Data-sharing statement

Data are available for download from dbGaP: https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs002504.v1.p1.

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