Germline bi-allelic *SH2B3*/LNK alteration predisposes to a neonatal juvenile myelomonocytic leukemia-like disorder

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Abstract

Juvenile myelomonocytic leukemia (JMML) is a rare, generally aggressive myeloproliferative neoplasm affecting young children. It is characterized by granulomonocytic expansion, with monocytosis infiltrating peripheral tissues. JMML is initiated by mutations upregulating RAS signaling. Approximately 10% of cases remain without an identified driver event. Exome sequencing of two unrelated cases of familial JMML of unknown genetics and analysis of the French JMML cohort identified 11 patients with variants in *SH2B3*, encoding LNK, a negative regulator of the JAK-STAT pathway. All variants were absent from healthy population databases, and the mutation spectrum was consistent with a loss of function of the LNK protein. A stoploss variant was shown to affect both protein synthesis and stability. The other variants were either truncating or missense, the latter affecting the SH2 domain that interacts with activated JAK. Of the 11 patients, eight from five families inherited pathogenic bi-allelic *SH2B3* germline variants from their unaffected heterozygous parents. These children represent half of the cases with no identified causal mutation in the French cohort. They displayed typical clinical and hematologic features of JMML with neonatal onset and marked thrombocytopenia. They had a hypomethylated DNA profile with fetal characteristics and did not have additional genetic alterations. All patients showed partial or complete spontaneous clinical resolution. However, progression to thrombocythemia and immunity-related pathologies may be of concern later in life. Bi-allelic *SH2B3* germline mutations thus define a new condition predisposing to a JMML-like disorder, suggesting that JAK pathway deregulation is capable of initiating JMML, and opening new therapeutic options.

Introduction

Juvenile myelomonocytic leukemia (JMML) is a rare, aggressive myeloproliferative neoplasm affecting in-fants and young children. It is characterized by excessive granulomonocytic proliferation in bone marrow (BM) and peripheral blood (PB) leading to splenomegaly, leukocytosis with precursors in PB, monocytosis, infiltration of

peripheral tissues with histiocytes and normal or moderately increased blast count.[1,2]

The natural course of JMML is generally rapidly fatal. The only potentially curative treatment is BM transplantation. However, the disease is characterized by a highly heterogeneous course, with a third of patients progressing to acute leukemia, while about 10% have indolent forms or even spontaneous resolutions.[3]

JMML arises from hematopoietic stem or myeloid progenitor cells.[4-6] It is initiated by abnormal activation of RAS signaling, leading to *in vitro* hypersensitivity of myeloid progenitors to granulocyte-macrophage colony-stimulating factor.[7] RAS pathway hyperactivation is due to mutations in genes encoding RAS proteins (*NRAS, KRAS*) or regulators (*PTPN11, NF1* or *CBL*),[8,9] which define genetic and clinical subgroups. More rarely, activating mutations affecting other small G proteins (RRAS, RRAS2, RIT1)[10] have also been described, as well as fusions causing activation of genes coding for transducers upstream of the RAS pathway.[11,12]

A particular feature of JMML is its frequent occurrence in the context of a predisposing genetic syndrome, including Noonan syndrome, neurofibromatosis type 1 and CBL syndrome, which are due to a constitutional upregulation of the RAS-MAPK pathway (the so-called RASopathies).[13-16] The identification of an activating mutation affecting the RAS pathway confirms the diagnosis of JMML in over 90% of cases.[17] Nevertheless, there are still a small number of patients in whom no mutation has been identified (e.g., 5% in the French JMML cohort).

SH2B3 (OMIM 605093) encodes LNK (lymphocyte adaptor protein), an SH2-domain adaptor protein that acts as a negative regulator of intracellular signaling promoted by cytokines and growth factors activating the JAK-STAT pathway.[18] Earlier work suggested that Lnk negatively regulates normal hematopoietic stem and progenitor cell expansion and self-renewal.[19,20] Somatic *SH2B3* variants have been reported in some JMML cases[21] and *Sh2b3*^{-/-} mice develop a myeloproliferative neoplasm.[22,23] Germline mono-allelic,[24-27] or more rarely bi-allelic[28,29] LNK loss-of-function mutations have been previously identified in various hematologic diseases.

Here we provide evidence that bi-allelic germline inactivating variants in *SH2B3* underlie a disease predisposing to a JMML-like disorder. We report 11 patients with *SH2B3*-associated JMML, including eight from five families who inherited bi-allelic pathogenic germline *SH2B3* variants. *SH2B3*-associated JMML represents a new specific entity characterized by extremely early onset, probable persistence of fetal features, and partial or complete spontaneous clinical resolution. JMML in this group of patients is clearly distinct from other forms of JMML as a whole, but also from the disease associated with germ-line mono-allelic or somatic mutations of SH2B3 that have previously been reported.

Methods

Patients

The study included 234 patients with JMML referred to the French reference laboratory between 1995 and 2023, and one family with two probands from the Bambino-Gesù Children's Hospital (OPBG) in Rome, Italy.

The diagnosis of JMML was based on clinical and hematologic findings, centrally reviewed cytomorphological examination of PB and BM smears, and genetic screening of genes known to initiate JMML or differential diagnoses (Wiskott-Aldrich syndrome, *GATA2*, osteopetrosis). Absence of *BCR::ABL1* and *KMT2A* rearrangements was checked. In patients with no RAS-related variant, the presence of rare fusions involving *FLT3, ALK,* and *PDGFRA/B* was assessed by RNA-sequencing analysis. Karyotyping was performed using standard procedures. All patients fulfilled the World Health Organization's criteria for JMML.

Ethical approval

The study was approved by the institutional review board of "Hôpitaux Universitaires Paris Nord Val-de-Seine" in Paris (Ref: 00006477) and the OPBG in Rome (Ref: 1702_OPBG_2018), in accordance with the Helsinki declaration.

Samples

BM or PB samples were obtained from the patients at the time of diagnosis of JMML and from the patients' parents. Mononuclear cells were isolated on a Ficoll gradient (Eurobio). The constitutional origin of the variant(s) was assessed using skin-derived fibroblasts. Healthy controls used for methylation and RNA-sequencing assays were males of 9, 10 and 12 years of age. The fetal BM sample was obtained from the product of a spontaneous abortion at 15 weeks of gestation. *Online Supplementary Figure S1* shows the analyses performed in patients.

Genome-wide DNA array analysis

Genomic DNA samples were analyzed by single-nucleotide polymorphism array technologies using the Genome-Wide GeneChip Human SNP Array 6.0 (Affymetrix), and/or high-density array comparative genomic hybridization.[12]

Whole-exome sequencing and whole-genome sequencing

Target enrichment was performed using the SureSelect Human All Exon V4+UTRs or SureSelect AllExon V5 (Agilent Technologies, Santa Clara, CA, USA), and captured regions were sequenced with a HiSeq2000 or NextSeq500 instrument (Illumina, San Diego, CA, USA), as previously described.[12,33] Details are provided in the *Online Supplementary Material*.

Sequencing the mRNA of mononucleated cells

Libraries were prepared with NEBNext Ultra II Directional

RNA Library Prep Kit, according to the supplier's recommendations. Paired-end 100-bp read sequencing was performed on a NovaSeq platform. Image analysis and base calling were performed using Illumina Real Time Analysis (3.4.4) with default parameters. The bioinformatic pipeline is described in the *Online Supplementary Information*. Unsupervised analyses of gene expression and differential expression were conducted using the Galileo tool (IntegraGen), as described in the *Online Supplementary Information*.

DNA methylation (capture enzymatic methyl sequencing)

Libraries were prepared using the Twist Targeted Methylation sequencing protocol system (Twist Bioscience) according to the guidelines and subjected to paired-end sequencing with 100-bp reads on a Novaseq 6000 platform (*Online Supplementary Information*). An unsupervised classification based on the 1,000 most variant 100 bp tiles was generated using hierarchical clustering (cosine distance, Ward method) in R software.

Further information

More details on the methods are provided in the *Online Supplementary Material*.

Results

Exome sequencing in two families segregating JMML identifies homozygous mutations in *SH2B3*

Trio exome sequencing was undertaken in proband #79.1 from the French JMML cohort, who suffered a syndromic neonatal JMML with no identified mutation. The analysis revealed the presence of a homozygous variant (c.1160G>C, p.Gly387Ala) in *SH2B3* in the affected child. A review of the child's records confirmed that he met the consensus clinical and hematologic criteria for the diagnosis of JMML, with hepatosplenomegaly, massive monocytosis (14×10^9/L), blasts, circulating myeloid precursors and thrombocytopenia (platelet count, 21×10^9/L) (Figure 1A; *Online Supplementary Table S1*). The BM smear showed hypercellularity associated with a decreased number of megakaryocytes. Spontaneous *in vitro* growth of myeloid progenitors was positive. The child's brother (#79.2), born a few years later, also presented with neonatal JMML (Figure 1). Genetic analysis showed that he had inherited the familial *SH2B3* mutation in a bi-allelic pattern.

Independently, whole-exome sequencing was performed in a family having two siblings diagnosed with an unclassified neonatal-onset syndromic JMML at the OPBG (Rome, Italy). The proband, #OPBG-2, was diagnosed at the age of 1 month, with elevated leukocyte counts, monocytosis (4.2×10^9/L), thrombocytopenia (platelet count, 41×10^9/L) and 3% myeloblasts (Figure 1B; *Online Supplementary Ta-*

ble S1). Immature monocytes and mild dysgranulopoiesis were observed in PB. Hypercellular BM, with granulocytic expansion, and 7% blasts, were consistent with a diagnosis of JMML. Similarly, his 5-year-old sister, #OPBG-1, had been diagnosed with JMML in the first days of life. Following delivery, she had splenomegaly and respiratory difficulties, which necessitated 24 h intubation. Her blood count showed leukocytosis (white blood cell count, 56×10^9/L), anemia (hemoglobin, 11.8 g/dL), and thrombocytopenia (platelet count, 35×10^9/L) (*Online Supplementary Table S1*). She experienced spontaneous resolution under active surveillance. Hypersensitivity of myeloid progenitor cells to granulocyte-macrophage colony-stimulating factor was observed in both children. Whole-exome analysis revealed the presence of a homozygous frameshift variant (c.1709dupA, p.Asn570LysfsTer82) in *SH2B3* in both siblings.

Targeted sequencing of *SH2B3* in the whole French JMML cohort

SH2B3 sequencing was then extended to the 234 patients of the French JMML cohort. Filtering out variants with a frequency >10^{-4} in the general population (GnomAD Non cancer v.2.1.1 database), at least one variant was identified in nine of the 234 (3.8%) patients, including six of the 12 (50%) mutation-negative cases (*Online Supplementary Figure S2*). Searching for mutations in patients' fibroblasts and/or in their parents revealed that, in eight of the nine patients, the *SH2B3* variants identified were germline. Of these eight patients, six (from 4 families) carried bi-allelic *SH2B3* variants (Figure 1C). In the remaining two patients, the germline *SH2B3* variant was mono-allelic. Whole-genome sequencing was performed and ruled out an alteration of the second *SH2B3* allele.

Variant segregation in six families showed that germline variants were always inherited from unaffected heterozygous parents (Figure 1C). In four families, all affected children inherited bi-allelic *SH2B3* variants, consistent with an autosomal recessive transmission of JMML predisposition. Three of these families were consanguineous. In family #48, the mono-allelic germline *SH2B3* variant in the affected child was inherited from the unaffected father.

Only one of the 234 patients (#209) had an exclusively somatic *SH2B3* variant.

Assessing variant pathogenicity

Including the Italian family, a total of seven different *SH2B3* variants were identified. Among them, three were truncating (frameshift indels or stop codon), three were missense, and one caused protein elongation with a divergent C-terminus (Table 1; Figure 2A). Each variant was found as a single occurrence except p.Asp231Glyfs*39, which was found in two unrelated patients. All changes were predicted to be deleterious; none was referenced in the general population (Table 1) and, importantly, no homozygous truncating variant was referenced in gnomAD.

Based on the bi-allelic occurrence of variants, a loss-of-function effect was postulated as a functional consequence of the mutations identified. In line with this hypothesis, transient transfection experiments performed in 293T cells documented a dramatically reduced stability of the p.Asn570Lys*82 LNK mutant as compared to the wild-type protein (Figure 2B). Besides this effect at the protein level, assessment of *SH2B3* mRNA levels by real-time polymerase
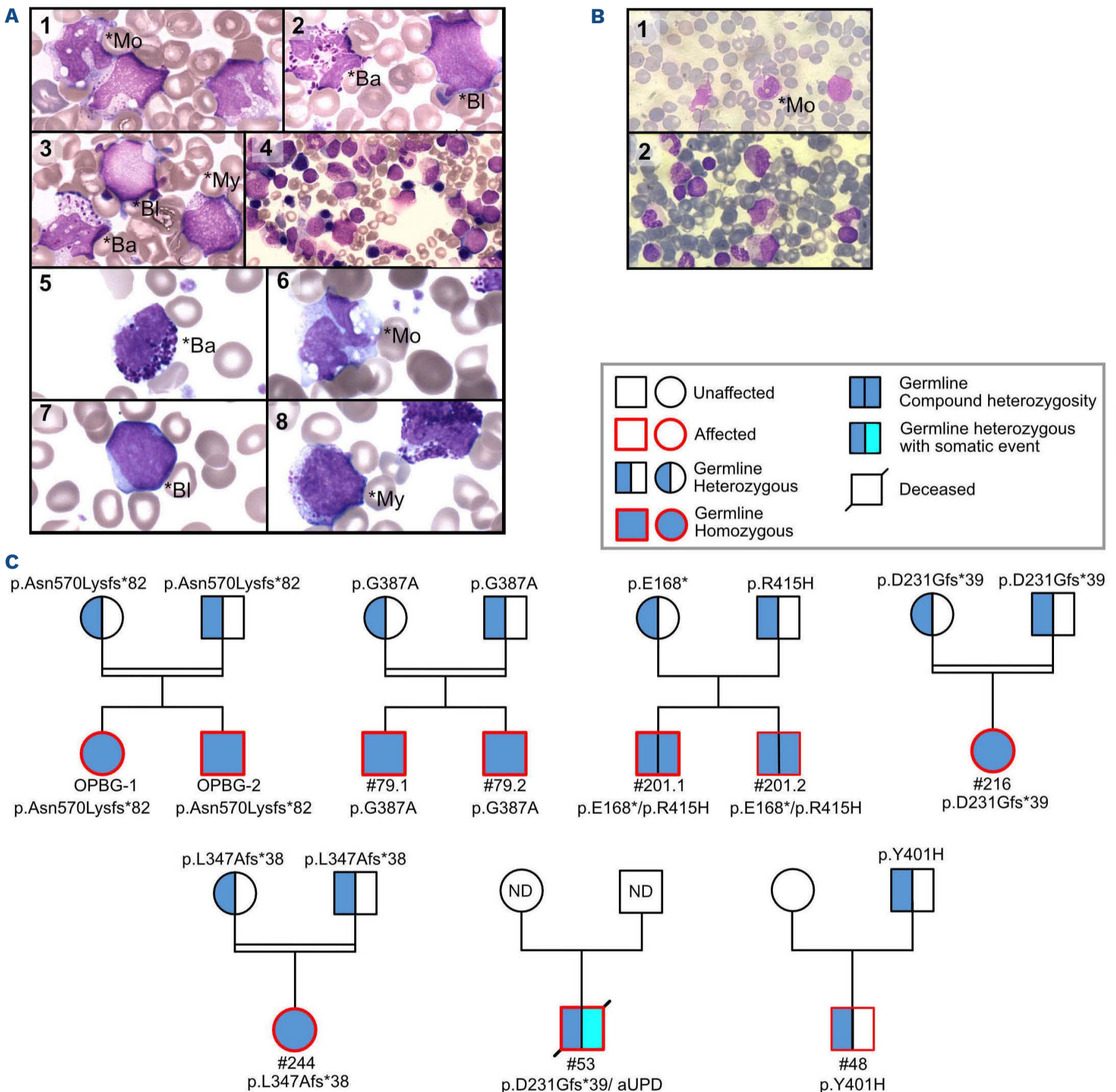

Figure 1. Patients' cytomorphological data and pedigrees. (A, B) May-Grünwald-Giemsa-stained smears showing cytomorphological features at diagnosis patients with of juvenile myelomonocytic leukemia (JMML). (A) Patients #79.1 (A1-4) and #79.2 (A5-8). (B) Patient #OPBG (B1, 2). Peripheral blood smears (A1-3; A5-8; B1) show monocytes (*Mo), myeloid precursors (*My), dysmorphic basophils (*Ba) and undifferentiated myeloid blasts (*Bl). Bone marrow smears (A4; B2) show hypercellularity with myeloid blasts and discrete signs of dysgranulopoiesis. The erythrocyte lineage is decreased and the megakaryocytic lineage is absent. (C) Pedigrees of patients with germline *SH2B3* variants. All children, but none of the heterozygous parents, had JMML. Germline variants (dark blue) were either bi-allelic (full circle/square; families 1 to 4) or mono-allelic (half circle/square; families 5 and 6) in the affected child. Somatic alteration is indicated in light blue. aUPD: acquired uniparental isodisomy; JMML: juvenile myelomonocytic leukemia; ND: not done.

Table 1. Germline and somatic *SH2B3* variants identified in patients with juvenile myelomonocytic leukemia (NM_005475.3).

Genomic coordinate (hg38)	Nucleotide change	Protein change	rs number	COSMIC	Description	MAF% (GnomAD)	REVEL score	CADD PHRED score	Predicted consequence/functional behavior
chr12:g.111856451G>T	c.502G>T	p.Glu168*	-	-	Truncating	0	-	33	Protein loss
chr12:g.111418830_111418836dup	c.685_691dupGGCCCCG	p.Asp231Glyfs*39	-	COSM9361236	Truncating	0	-	NA	Protein loss
chr12:g.111447346dup	c.1038dupG	p.Leu347Alafs*38	-	COSM4170093	Truncating	0	-	NA	Protein loss
chr12g.111447468G>C	c.1160G>C	p.Gly387Ala	-		Missense (SH2)	0	0.884	28.5	Structural damage
chr12:g.111447509T>C	c.1201T>C	p.Tyr401His	rs1426386395		Missense (SH2)	0	0.958	28.5	Structural damage
chr12:g.111447663G>A	c.1244G>A	p.Arg415His	rs918140013	COSM6228211	Missense (SH2)	0	0.542	28.4	Structural damage/impaired target binding and reduced ability to inhibit signaling (Morris, Nat Com 2021)
chr12:g111886085 C>CA	c.1709dupA	p.Asn570LysfsTer82	-	-	Stoploss	0	-	33	Decreased transcript stability/processing and protein stability (this study)

COSMIC: Catalog of Somatic Mutations in Cancer; MAF: minor allele frequency; REVEL: rare exome variant ensemble learner; CADD: Combined Annotation Dependent Depletion; NA: not applicable.
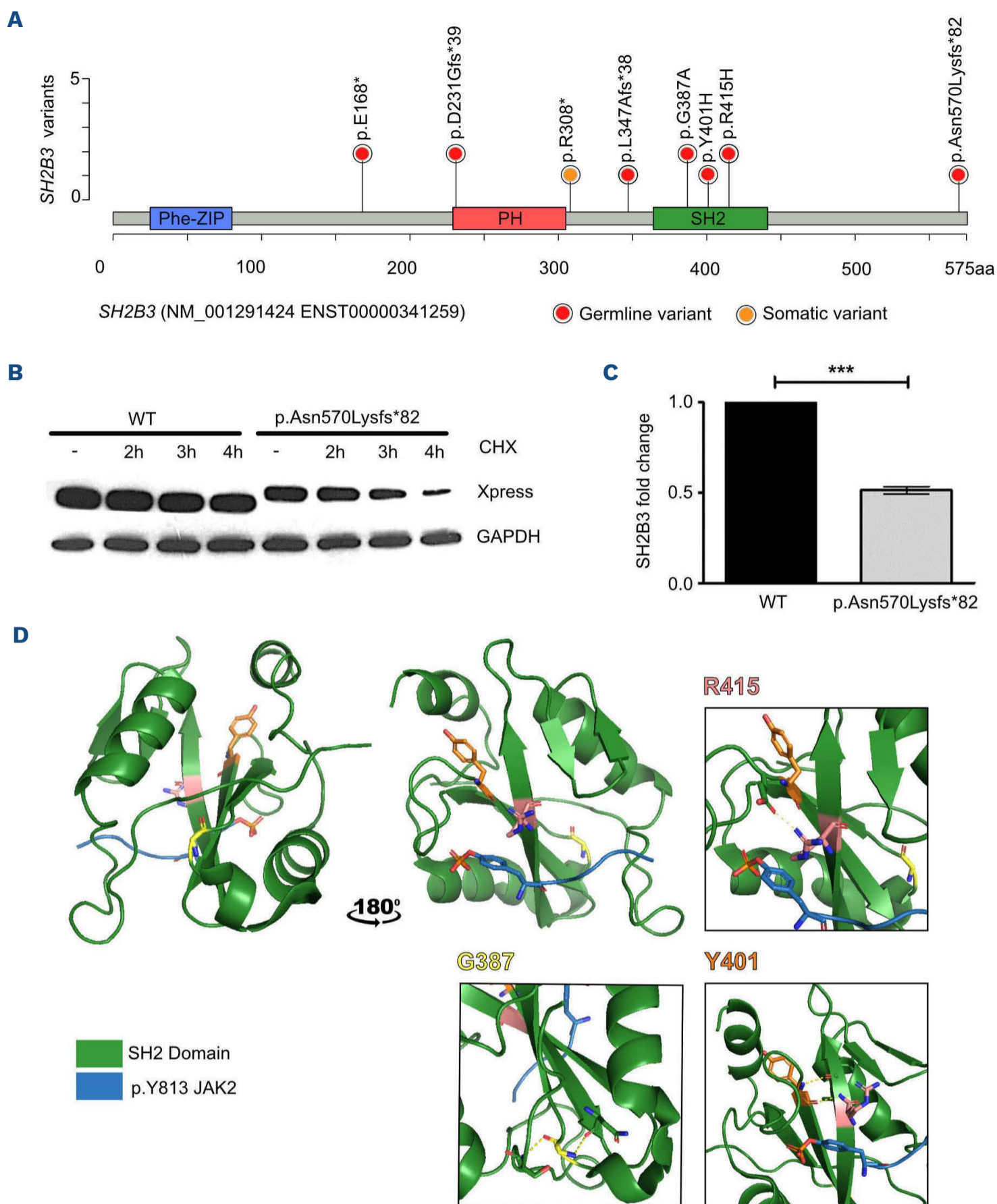
Figure 2. *SH2B3* variants and consequences on the LNK protein. (A) Lollipop plot showing the distribution of *SH2B3/*LNK variants over a schematic representation of the LNK protein. The PH domain enables interaction with cell membrane phospholipids and the SH2 domain binds to the phosphorylated tyrosines of target proteins. (B, C) A disease-causing *SH2B3* stoploss frameshift mutation affects RNA and protein stability. (B) Protein stability was assessed in 293T cells transiently transfected with wild-type Xpress-tagged *SH2B3* and mutant carrying the c.1709dupA (p.Asp570Lysfs82) mutation. After transfection (48 h), cells were treated with cyclohex-imide (20 μg/mL) for the indicated time or left untreated. Protein levels were assessed by immunoblotting, using an anti-Xpress monoclonal antibody. GAPDH levels are shown to document equal loading of total proteins from cell lysates. Western blot from a representative experiment of three performed is shown. (C) Quantitative polymerase chain reaction analysis performed on RNA extracted from fibroblasts taken from patients and controls shows a significant decrease of *SH2B3* expression level indicating RNA decay of the allele carrying the *SH2B3* variant. Data in the graph indicate fold change of *SH2B3* in patients' fibroblasts over that in control cells (WT), set as 1. *GAPDH* was used as an endogenous control. Histograms show mean values ± standard deviation of three independent experiments, each performed in triplicate. The analysis of expression was performed calculating the fold change using the 2−ΔΔCt formula and the results were statistically analyzed by PRISM7, using a two-tailed unpaired *t* test with the Bonferroni correction. ***$P<0.001$. (D) Three-dimensional structure of the SH2 domain of the LNK protein with the JAK2 phosphopeptide pY813 modeled from crystallographic data in the report from Morris *et al.*[36] (PDB:7R8W). The position of variant amino acids was deter-mined by structural homology between mouse and human SH2 domains. aa: amino acids; CHX: cycloheximide; WT: wild-type.

chain reaction in primary fibroblasts obtained from patient #OPBG-2 and an unaffected control provided evidence of a significantly reduced amount in the former, which was suggestive of reduced stability of the transcript carrying the variant (Figure 2C). Overall, these findings indicate a disruptive impact in terms of transcript stability/processing and protein stability. Similarly, all missense variants were found to target the SH2 domain, which mediates binding to phosphorylated JAK and contains only rare polymorphisms in the general population (*Online Supplementary Figure S3*). All three variants were predicted to damage the structure of the SH2 domain of the LNK protein (Figure 2D), likely resulting in a decreased capacity to bind its JAK2 target. Collectively, these findings are consistent with a loss of function of *SH2B3* as a driver mechanism underlying JMML.

Hematologic and clinical features of patients with *SH2B3* mutations

All patients presented with splenomegaly, high monocyte counts and hematologic data meeting the updated World Health Organization's consensus criteria for the diagnosis of JMML[31] (*Online Supplementary Table S1*). Patients with bi-allelic germline mutations (n=8) had higher counts of white blood cells (*P*<0.001), monocytes (*P*=0.045) and lymphocytes (*P*=0.004) than those with mono-allelic/somatic (n=3) variants or patients from the whole JMML cohort (Table 2). This also held true when these patients were compared to patients with Noonan syndrome-associated JMML (Table 2) or with any of the genetic subgroups of JMML taken separately.[9] Notably, all patients with *SH2B3* alterations but one displayed marked thrombocytopenia and the median platelet count was significantly lower than in the whole JMML cohort (28×10^9/L *vs.* 64×10^9/L; *P*=0.02), with frequent lack of megakaryocytes in patients' BM (*Online Supplementary Table S1*). BM cellularity was normal or increased, with predominant granulocytic proliferation. Mild dysplastic features were observed in about half of the patients. Blast cells were moderately elevated but lower

Table 2. Clinical and hematologic features of patients with *SH2B3*-mutated juvenile myelomonocytic leukemia (JMML) in comparison with patients with Noonan syndrome-JMML[47] (Strullu *et al.*, updated) and with the whole French JMML cohort (excluding patients with Noonan syndrome).

Parameters	*SH2B3* GL bi-allelic	*SH2B3* GL/somatic	Patients with NS	Total cohort without NS
N of cases	8	3	33	161
Male:female ratio	1	3	1.8	1.5
Age in months at onset of hematologic anomalies, median (range)	0.3 (0-2)	45.6 (26.4-52.8)	1.3 (0-14.9)	20.9 (0.7-188.4)
Peripheral blood				
Hemoglobin concentration, g/dL, median (range)	11.3 (6.7-19.9)	10.5 (10.1-11.2)	12.1 (6.6 – 21.9)	9.3 (3.5-13.8)
Platelet count ×10^9/L , median (range)	28 (13-289)	23 (18-93)	126 (19-430)	64 (5-428)
WBC count ×10^9/L, median (range)	61.5 (46-81.6)	29.4 (11.7-71.2)	23.6 (3.2-112)	25.0 (4.6-168)
Monocyte count ×10^9/L, median (range)	7.58 (2.8-19.6)	2.6 (2.06-17)	4.9 (0.6-24.6)	4.9 (0.9-38)
Lymphocyte count ×10^9/L, median (range)	16.9 (6.9-40)	5.0 (3.16-12.8)	7.4 (2-37)	7.8 (0.8-42)
Myeloid precursors in peripheral blood, N of patients	8/8	3/3	21/24	118/137 (86)
Blasts in peripheral blood, median % (range)	4.0 (0-6)	3.0 (1-17)	0.0 (0-8.5)	1.0 (0-27)
Bone marrow				
Blasts in bone marrow, median % (range)	4.5 (2-11)	6.0 (2-12)	3.0 (0-9)	4.0 (0-73)
Dysplastic features, N/N of patients (%)	5/8	3/3	16/26	36/49 (73)
Decreased erythroid lineage, N/N of patients (%)	7/8	1/3	9/26	9/49 (18)
Decreased megakaryocytes, N/N of patients (%)	5/8	1/3	7/26	27/49 (55)
Clinical presentation, N/N of patients (%)				
Splenomegaly	8/8	3/3	23/33	149 (94)
Hepatomegaly	7/8	2/3	20/33	120 (75)
Adenopathy	4/4	1/3	1/29	87 (55)
Skin lesions	0/8	1/3	4/29	55 (38)
Respiratory symptoms	1/8	1/3	15/29	22 (16)
Bleeding	2/8	1/3	1/29	32 (24)
Secondary genetic alteration, N/N of patients (%)	0/8	3/3	0/31	74/152 (49)
Treatment and follow-up, N/N of patients (%)				
Chemotherapy	4/8	3/3	6/28	-
Bone marrow transplantation	1/8	3/3	2/31	118/161 (73)
Clinical outcome (alive)	8/8	2/3	20/31	111/161 (68)

GL: germline; NS: Noonan syndrome; N: number; WBC: white blood cell.

than 20% in blood and BM.

In patients with bi-allelic germline mutations, JMML onset was neonatal. Most patients (5/6) were not transplanted and had spontaneous resolution.

In striking contrast, the median age of onset of JMML with mono-allelic germline or somatic *SH2B3* variants was 3.8 years, which is later than patients with bi-allelic germline *SH2B3* variants. The former had a markedly more severe course compared to the latter, and have all undergone transplantation.

Longitudinal follow-up of patients with bi-allelic *SH2B3* germline variants shows that after an initial period of thrombocytopenia associated with profound impairment of the megakaryocytic lineage, the children's platelet counts increased rapidly, reaching above-normal levels in several of them, with even thrombocytosis in patient #79.2 (*Online Supplementary Figure S4*).

Genetic makeup of *SH2B3* mutated JMML

To determine the mutational makeup of JMML associated with *SH2B3* variants, genome-wide DNA array analysis and high-depth sequencing of recurrently mutated genes in JMML were carried out. Whole-genome sequencing was also performed on patients with available germline samples (*Online Supplementary Figure S1*). All patients with bi-allelic *SH2B3* variants had a normal karyotype and none had deleterious variants in known JMML drivers. Extensive genetic analysis did not evidence any other germline or somatic pathogenic or likely pathogenic variant or copy number alteration (Figure 3).

In contrast, patients with a mono-allelic germline *SH2B3* variant acquired several additional somatic variants. Regardless of their germline or somatic status, mono-allelic *SH2B3* mutations were systematically associated with a *PTPN11* mutation (Figure 3).
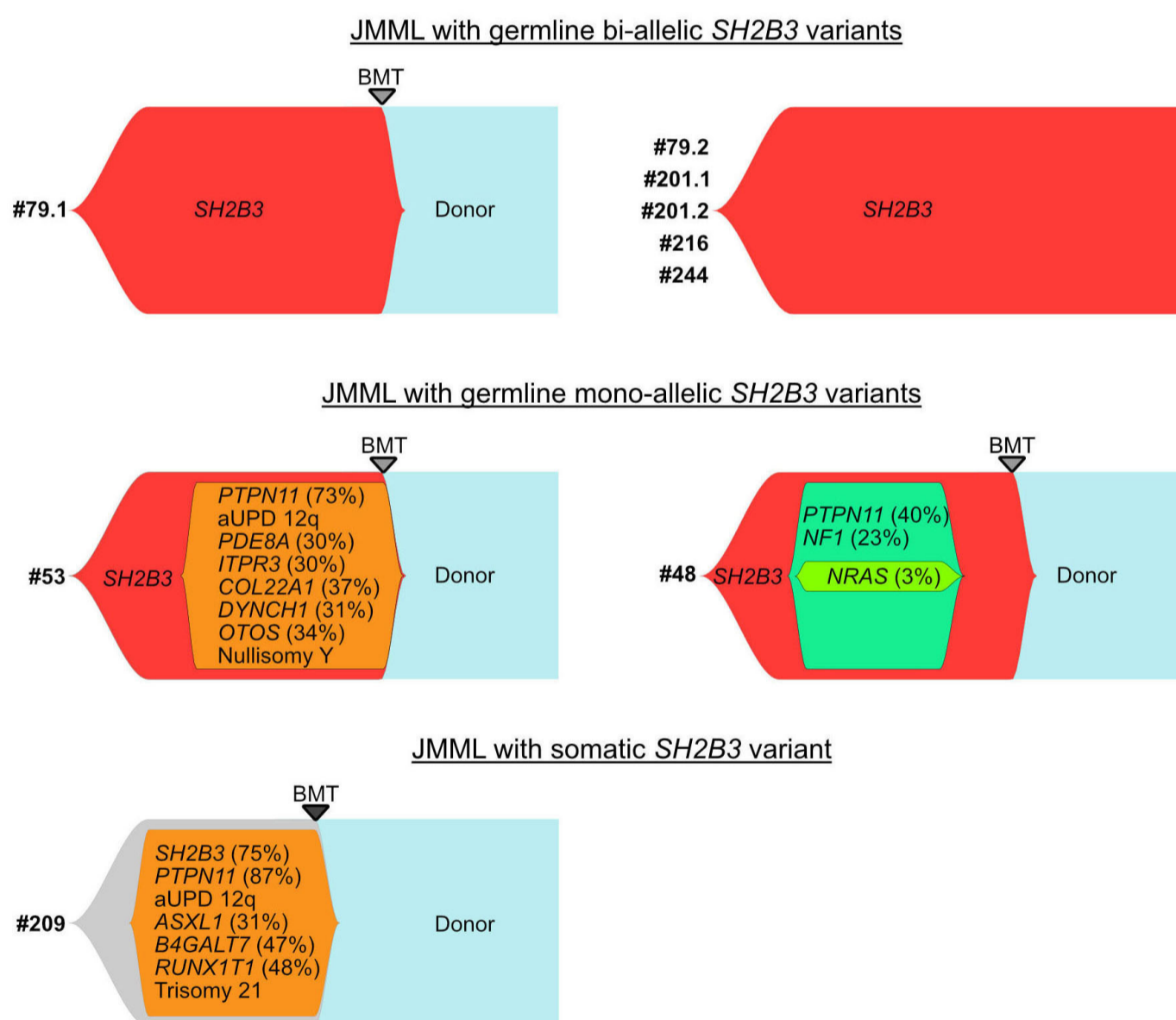

Figure 3. Clonal architecture of *SH2B3* mutated juvenile myelomonocytic leukemia. Patients with a bi-allelic germline mutation of *SH2B3* (red clones) (#79, #201, #216, #244) all have a monomorphic profile with no additional genetic alterations. Only patient #79.1 received a bone marrow transplant (upper panel). Patients with a germline mono-allelic *SH2B3* mutation (#53, #48) and/or somatic *SH2B3* mutation (orange clones) acquired several additional somatic mutations. Patient #53 had a somatic *PTPN11* variant and patient #48 had somatic variants of *PTPN11* and *NF1*, whose order of appearance could not be inferred from the allelic frequencies. Both patients underwent bone marrow transplantation (middle panel). In patient #209, the allelic frequency of the somatic *SH2B3* variant was consistent with its presence in the full leukemia clone. Multiple other somatic alterations were acquired, including a driver somatic mutation in *PTPN11*. Here again, all potentially driver mutations were present at variant allele frequencies consistent with early co-occurrence during the course of the disease and order or appearance could not be determined (lower panel). JMML: juvenile myelomonocytic leukemia; BMT: bone marrow transplant; aUPD: acquired uniparental isodisomy.

Notably, two patients (#53; #209) with a mono-allelic *SH2B3* truncating variant and a *PTPN11* mutation subsequently acquired chromosome 12q uniparental disomy (12q21.1-q24.31 and 12q13.2-q24.31, respectively), resulting in copy-neutral loss of heterozygosity of the *SH2B3* variants, but also of *PTPN11*, which is located on chromosome 12q24.13, close to *SH2B3* (12q24.12). Such a pattern of events is rarely found in patients with *PTPN11* mutations, which have a gain-of-function behavior, but is a typical mechanism for the complete inactivation of tumor suppressor genes. In our patients, it leads to the bi-allelic loss of function of LNK. Taken together, this supports the view that the loss of function of *SH2B3* is a driving event in JMML in these cases as well. It is likely that the proximity to *PTPN11* is the reason why a copy-neutral loss of heterozygosity is privileged over a simple deletion of *SH2B3* in these cases.

DNA methylation profiling

DNA methylation was also analyzed. A reference group of 54 cases of JMML of known genetic groups and two samples (one postnatal BM, one prenatal BM) from healthy subjects were used for comparison. As expected, hierarchical clustering of reference samples delineated three main groups, corresponding to methylation-low, intermediate and high JMML subtypes. The patients with a mono-allelic germline (#53) or somatic (#209) *SH2B3* mutation clustered within the intermediate and hypermethylated groups, respectively. In contrast, samples with bi-allelic germline *SH2B3* variants defined a distinct cluster (cluster 1) with the strongest hypomethylation pattern (Figure 4A). Interestingly, the prenatal BM clustered with the *SH2B3* cluster whereas the postnatal BM clustered in cluster 3 (Figure 4A).

Altogether, methylation profiling is consistent with the idea that JMML with bi-allelic germline *SH2B3* mutations represents a specific entity that may be linked to prenatal features, in line with the neonatal presentation.

RNA sequencing of JMML mononucleated cells

Total RNA sequencing was performed on mononuclear cells obtained at diagnosis from four patients with bi-allelic germline *SH2B3* mutations. The gene expression profile was compared to that of 17 reference JMML samples and two postnatal BM samples from healthy subjects. Principal component analysis showed that the four *SH2B3* cases clustered within the group of JMML samples (Figure 4B). Analysis of differential gene expression between *SH2B3* JMML and postnatal BM samples evidenced 447 genes overexpressed, compared with 717 genes underexpressed, in *SH2B3* JMML cases (Figure 4C). Interestingly, the oncofetal transcript *LIN28B* and its two targets *HBG2* and *IGF2BP1* were among the most overexpressed genes in *SH2B3* JMML samples. When compared with other JMML cases, the number of overexpressed genes fell to only 13, with the oncofetal transcript *IGF2BP1* ranking first (Figure 4D).

Discussion

SH2B3 encodes the lymphocyte adaptor protein LNK, a member of the SH2B adaptor family of proteins. LNK is predominantly expressed in hematopoietic stem and progenitor cells and is a key negative regulator of numerous cytokine and growth factor receptors.[36]

We show here that bi-allelic germline loss-of-function *SH2B3* mutations represent a new disease predisposing to a JMML-like disorder with autosomal recessive inheritance. In our cohort, it accounted for 50% of JMML cases with unsolved genetics, and all cases experienced spontaneous resolution, in line with a hypomethylated DNA pattern indicative of a favorable prognosis.[37]

JMML-associated germline *SH2B3* mutations were frameshift or missense targeting the SH2 domain. The SH2 domain is required for LNK to bind to the phosphorylated tyrosine (pY813) of JAK2 or JAK3 when activated in response to cytokines. Variants targeting the SH2 domain have been reported to cause severe disruption to LNK function.[20] All variants were thus consistent with loss of function of the protein. Deficiency of LNK leads to increased JAK/STAT signaling in a cytokine-independent manner,[36] and mice deficient in LNK were demonstrated to have a variety of hematopoietic phenotypes, including expansion of the hematopoietic stem and progenitor cell compartment,[19,38] which is consistent with JMML.[5,6] It is now known that LNK controls the expansion of hematopoietic stem cells and myeloid progenitors predominantly by modulating the thrombopoietin signaling pathway.[20] Indeed, thrompoietin, which was initially identified as the cytokine that stimulates megakaryopoiesis and platelet production, has also been shown to be a major regulator of hematopoietic stem cell self-renewal and quiescence.[20]

Both germline and somatic LNK loss-of-function mutations have been previously identified in a range of hematologic diseases.[24] Heterozygous *SH2B3* germline variants, some of which are relatively common in the general population, have been associated with a low penetrance increased risk of myeloproliferative neoplasms[25] and idiopathic erythrocytosis.[26,27] Homozygous pathogenic variants, by contrast, are absent from reference population databases and have scarcely been described in patients. The first case reported was that of a consanguineous family in which two siblings carrying D231Profs*38 presented with growth retardation, mild developmental delay and autoimmune disorders.[28] Although this was not explicitly presented as a JMML, the proband developed a phenotype very similar to that of our patients with high white blood cell count, <20% blasts, thrombocytopenia, and no megakaryocytes in BM at 4 weeks old. Blood cell counts normalized without intervention by the age of 14 months; however, the boy then developed liver cirrhosis alike to one of our patients.

More recently, two unrelated patients with homozygous germline loss-of-function *SH2B3* variants R148Profs*40
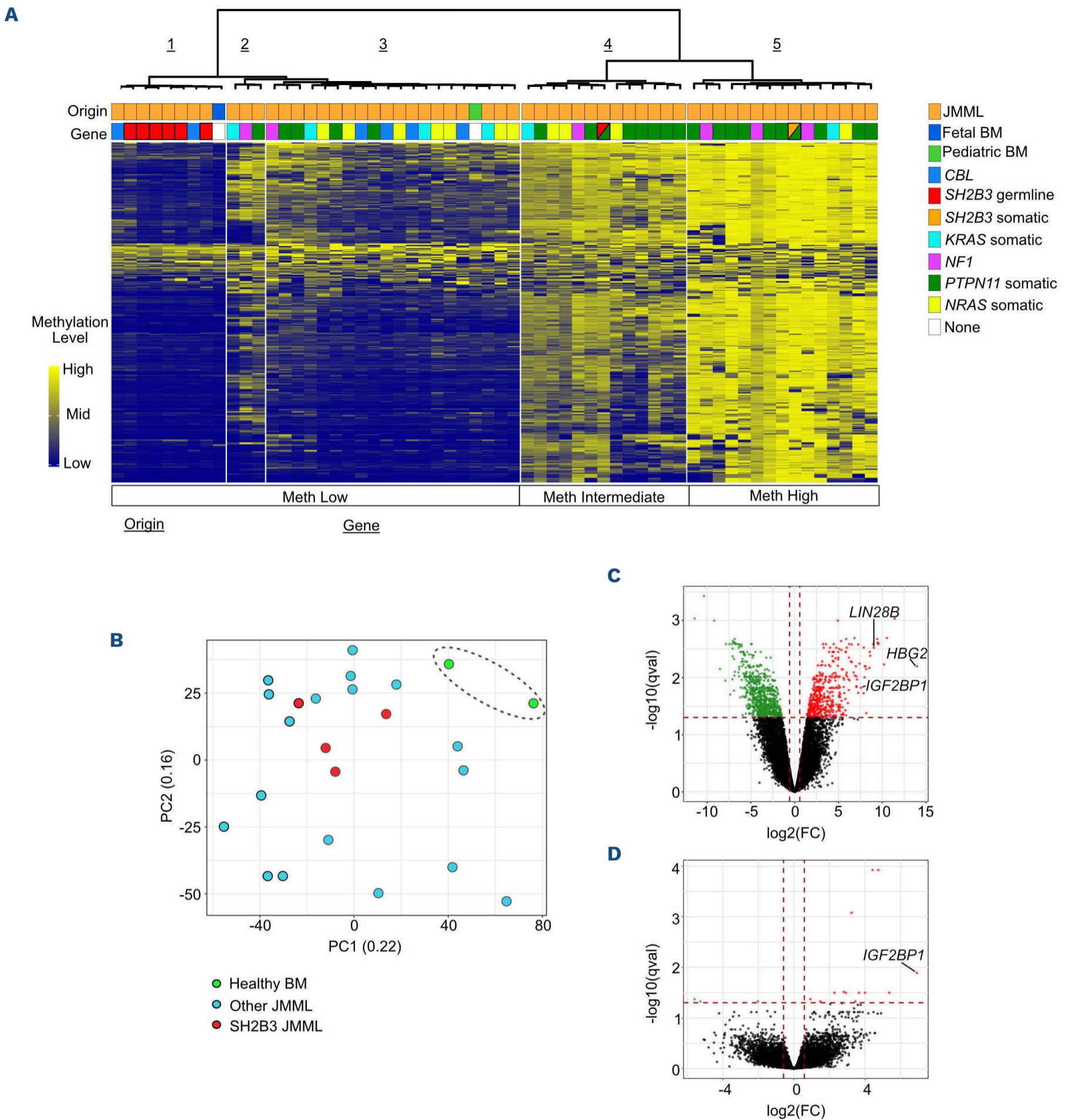
Figure 4. DNA methylation pattern and gene expression profiling suggest persistence of fetal cues in juvenile myelomonocytic leukemia with bi-allelic germline *SH2B3* variants. (A) DNA methylation analysis of patients with *SH2B3* variants (N=8) and reference juvenile myelomonocytic leukemia (JMML) of known genetic groups (i.e., *PTPN11* somatic, *NRAS* somatic, *KRAS* somatic, *NF1* or *CBL*) (N=54). Unsupervised hierarchical clustering based on the 1,000 most variant 100 bp tiles identified three main DNA methylation subgroups (low, intermediate, high). JMML with bi-allelic *SH2B3* germline mutations defines a subgroup of hypomethylated JMML (cluster 1), whereas JMML with either mono-allelic or somatic *SH2B3* mutations cluster in the intermediate (cluster 4) or high (cluster 5) methylation groups. (B-D) Gene expression profile analyses of mononucleated cells of JMML with bi-allelic *SH2B3* germline alteration (N=4) in comparison with other types of JMML (N=17) and bone marrow samples from healthy subjects. Principal component analysis (B) and volcano plots showing differential gene expression between JMML with bi-allelic *SH2B3* germline alteration and healthy bone marrow (C) or other types of JMML (D). A qval threshold of ≤0.05 and a minimum fold-change of 1.5 were used to define differentially expressed genes. JMML: juvenile myelomonocytic leukemia; BM: bone marrow; Meth: methylation; PC: principal component; FC: fold change.

and V402M with developmental delay, hepatosplenomegaly, myeloproliferation and autoimmune disorders were described.[29] Paradoxically, unlike other patients, a major thrombocytosis with megakaryocytic hyperplasia was noted in the BM of these patients. Similarly, even though our patients initially presented with pronounced thrombocytopenia they progressed after spontaneous resolution of JMML towards having thrombocytosis. This could therefore represent two successive phases of the same disease.

How LNK-deficient patients progress from a major defect in platelet production to excess production is unclear. The effect of LNK loss on megakaryopoiesis may be age-dependent.[39,40] Indeed, the role of thrombopoietin appears to be different in fetal hematopoietic stem cells, which have a high capacity for proliferation and self-renewal for up to 6 months after birth, and in adult hematopoietic stem cells, which are maintained quiescent.[41] In this respect, it is interesting to note that all JMML in patients with bi-allelic germline *SH2B3* mutations had a neonatal onset and retained fetal characteristics, such as a DNA methylation pattern resembling that of fetal BM and expression of the oncofetal transcripts *LIN28B* and *IGF2BP1*.[42] The progressive loss of this fetal context may then explain the spontaneous regression of JMML and the switch to thrombocytosis.

RASopathies may be associated with immune disease,[43] and the combination of spontaneously resolving forms of JMML associated with a propensity to subsequently develop immune-related disorders is particularly reminiscent of what is observed in patients with CBL syndrome.[16] Interestingly, LNK recruits the CBL E3 ubiquitin ligase via its tyrosine residue at position 572 and thus inactivates JAK2 by degradation via the proteasome.[44] This functional link has recently been extended to RAS activation. Indeed, JAK2 stabilization induced by loss of function of CBL dramatically enhances palmitoylation of NRAS, thereby activating the RAS-MAPK signaling pathway and promoting myeloid leukemogenesis.[45] Such interplay between the JAK-STAT and RAS-MAPK signaling pathways highlights how the loss of LNK could induce JMML whose pathogenesis is considered to be strictly linked to activation of the RAS pathway.[46]

Besides immune-hematologic defects, several of our patients, as well as those reported in the literature,[28,29] had multiple extra-hematologic disorders suggestive of a syndromic presentation. However, these signs were inconstant and differed widely from one patient to another, even within a family. It is therefore difficult to incriminate loss of *SH2B3* function in these associated features, which could simply result from the increased risk of autosomal recessive disorders in a context of consanguinity.

Our analysis also identified JMML patients with mono-allelic *SH2B3* variants. Regardless of their germline or somatic status, these patients had a strikingly different presentation than that of patients with bi-allelic germline variants, with a later age of onset and a much more severe clinical course requiring BM transplantation. In line with the clinical presentation, the mutational make-up and DNA methylation profile of patients with *SH2B3* mutations differed greatly depending on the allelic pattern. Unlike patients with bi-allelic germline *SH2B3* mutation, for whom this alteration seems to represent the only initiating driver of the JMML-like disorder, patients with mono-allelic germline or somatic SH2B3 variants presented with multiple somatic alterations, systematically including a somatic variant in *PTPN11*, although we could not determine which lesion was the initiating driver.

In conclusion, our findings show that *SH2B3* can act as a driver in JMML in two ways, highlighting the importance of deregulation of the JAK-STAT pathway in this disease. On the one hand, mono-allelic *SH2B3* alterations, either germline or somatic, drive a severe 'classical' JMML associated with frequent loss of heterozygosity and additional somatic mutations. On the other hand, bi-allelic germline variants of *SH2B3* define a singular recessive clinical entity associated with a predisposition to develop a neonatal JMML-like disorder. This new entity is estimated to account for half of the cases of JMML that remained unresolved on the genetic level. Its biology and clinical course distinguish it clinically from cases of JMML associated with somatic or constitutional mono-allelic pathogenic *SH2B3* variants. The absence of secondary somatic changes is reminiscent of the JMML-like disorder seen in some patients with Noonan syndrome. However, apart from the neonatal presentation, none of the unique hematologic features of patients with germline *SH2B3* bi-allelic mutations is shared with Noonan syndrome-JMML.[47] Furthermore, all of our cases had a favorable outcome, which is less common in Noonan syndrome-JMML.[47] This, together with the immunological landscape, makes them clinically more similar to patients with CBL syndrome. Indeed, strikingly, all previously reported patients were also described to develop autoimmune disorders (thyroiditis, hepatitis) at a later age.[28,29] Similarly, although their follow-up is still short, one of the present patients developed autoimmune thyroiditis and another presented with a severe liver disorder requiring transplantation. Thus, in addition to the JMML-like disorder, this disease seems to cause serious immune disorders, which should be monitored to favor prompt management and effective care.

Disclosures

No conflicts of interest to disclose.

Contributions

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