Abstract: Noonan syndrome (NS) is a relatively common developmental disorder with a pleomorphic phenotype. Mutations causing NS alter genes encoding proteins involved in the RAS-MAPK pathway. We and others identified Casitas B-lineage lymphoma proto-oncogene (CBL), which encodes an E3-ubiquitin ligase acting as a tumor suppressor in myeloid malignancies, as a disease gene underlying a condition clinically related to NS. Here, we further explored the spectrum of germline CBL mutations and their associated phenotype. CBL mutation scanning performed on 349 affected subjects with features overlapping NS and no mutation in NS genes allowed the identification of five different variants with pathological significance. Among them, two splice-site changes, one in-frame deletion, and one missense mutation affected the RING domain and/or the adjacent linker region, overlapping cancer-associated defects. A novel nonsense mutation generating a v-Cbl-like protein able to enhance signal flow through RAS was also identified. Genotype-phenotype correlation analysis performed on available records indicated that germline CBL mutations cause a variable phenotype characterized by a relatively high frequency of neurological features, predisposition to juvenile myelomonocytic leukemia, and low prevalence of cardiac defects, reduced growth, and cryptorchidism. Finally, we excluded a major contribution of two additional members of the CBL family, CBLB and CBLC, to NS and related disorders.

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Molecular Diversity and Associated Phenotypic Spectrum of Germline CBL Mutations

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ABSTRACT: Noonan syndrome (NS) is a relatively common developmental disorder with a pleomorphic phenotype. Mutations causing NS alter genes encoding proteins involved in the RAS-MAPK pathway. We and others identified Casitas B-lineage lymphoma proto-oncogene (CBL), which encodes an E3-ubiquitin ligase acting as a tumor suppressor in myeloid malignancies, as a disease gene underlying a condition clinically related to NS. Here, we further explored the spectrum of germline CBL mutations and their associated phenotype. CBL mutation scanning performed on 349 affected subjects with features overlapping NS and no mutation in NS genes allowed the identification of five different variants with pathological significance. Among them, two splice-site changes, one in-frame deletion, and one missense mutation affected the RING domain and/or the adjacent linker region, overlapping cancer-associated defects. A novel nonsense mutation generating a v-Cbl-like protein able to enhance signal flow through RAS was also identified. Genotype–phenotype correlation analysis performed on available records indicated that germline CBL mutations cause a variable phenotype characterized by a relatively high frequency of neurological features, predisposition to juvenile myelomonocytic leukemia, and low prevalence of cardiac defects, reduced growth, and cryptorchidism. Finally, we excluded a major contribution of two additional members of the CBL family, CBLB and CBLC, to NS and related disorders.


KEY WORDS: CBL mutation-associated syndrome; Noonan syndrome; RAS-MAPK; genotype–phenotype correlations

Introduction

Noonan syndrome (NS; MIM #163950) is an autosomal-dominant, relatively common, and clinically variable condition with an estimated prevalence of one in 1,000–2,500 live births [Mendez and Opitz, 1985]. It is characterized by postnatal reduced growth, facial dysmorphism, and congenital heart defects (CHDs) [Allanson, 2007; van der Burgt, 2007; Roberts et al., 2013]. Although the facial dysmorphia changes with age, the most recurrent features consist of a broad forehead, hypertelorism, downslanting palpebral fissures, ptosis, epicantal folds, and low-set, posteriorly rotated ears with thick helices. Apart from Down syndrome, NS is the most common syndromic cause of CHDs [Burch et al., 1993; Marino et al., 1999; Shaw et al., 2007]. Cardiac involvement is present in up to 80% of affected individuals, with pulmonary valve stenosis, hypertrophic cardiomyopathy (HCM), and septal defects occurring most commonly. Other associated features include chest and spine defects, ectodermal anomalies, webbed/short neck, variable cognitive deficits, cryptorchidism, delayed puberty, lymphatic dysplasia, and a wide spectrum of hematologic abnormalities, including transient monocytosis, coagulation defects, and, rarely, certain malignancies of infancy and childhood [Tartaglia et al., 2003; Jongmans et al., 2011; Kratz et al., 2011; Aoki and Matsubara, 2013].

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NS is genetically heterogeneous. Extensive studies performed in the last decade demonstrated that all the genes implicated in NS and related phenotypes encode proteins with a role in the RAS signaling, with disease-causing mutations generally enhancing signal flow through the MAPK cascade but also perturbing signal flow through other RAS-mediated pathways [Tartaglia et al., 2011]. Germline missense, gain-of-function mutations in PTPN11, which encodes SHP2, a cytoplasmic protein tyrosine phosphatase that functions as a positive regulator of RAS signaling [Neel et al., 2003], occur in approximately 50% of NS subjects [Tartaglia et al., 2001; 2002]. Activating mutations in seven additional genes coding for transducers or modulatory proteins participating in this pathway (i.e., SOS1, KRAS, NRAS, RIT1, RAF1, BRAF, and MAP2K1) account for an additional one-fourth of cases [Tartaglia et al., 2011; Aoki et al., 2013]. Remarkably, mutations in the same genes and other functionally related loci have been reported to underlie a group of clinically related conditions, which have been collectively named “RASopathies” [Aoki et al., 2008; Tartaglia and Gelb, 2010; Rauen, 2013; Flex et al., 2014].

We and others recently reported that germline mutations in the Casitas B-lineage lymphoma proto-oncogene (CBL; MIM #165360) cause a previously unrecognized condition that resembles NS phenotypically and predispose to juvenile myelomonocytic leukemia (JMML; MIM #607785) (CBL mutation-associated syndrome, hereinafter) [Martinelli et al., 2010; Niemeyer et al., 2010; Pérez et al., 2010]. CBL encodes a RING finger E3 ubiquitin ligase that negatively regulates intracellular signaling, targeting receptor tyrosine kinases (RTKs) for internalization and recycling/degradation [Swaminathan and Tsygankov, 2006; Dikic and Schmidt, 2007]. On the other hand, CBL positively modulates signal traffic through its adaptor function. Somatically acquired CBL mutations occur with variable prevalence in myeloid malignancies, including JMML, and are generally observed as homozygous lesions due to loss of the wild-type allele by acquired isodisomy of the 11q23 chromosomal region [Kales et al., 2010]. Collected data indicate that disease-associated mutations are predominantly splice site or missense changes affecting exons 8 and 9, which encode the RING finger domain and/or the adjacent linker helix region (LHR) connecting this domain to the N-terminal tyrosine kinase binding (TKB) domain. In line with studies focused on cancer-associated CBL mutations [Sargen et al., 2007; Sanada et al., 2009; Martinelli et al., 2012], functional characterization of a panel of RASopathy-causing mutants documented that lesions act in a dominant-negative manner, affecting CBL-mediated receptor ubiquitination, and upregulate signal flow through RAS [Martinelli et al., 2010; Niemeyer et al., 2010; Brand et al., 2014].

CBL mutation-associated syndrome is characterized by phenotypic heterogeneity and variable expressivity. Here, we further investigated the molecular spectrum of germline CBL mutations, their impact on protein function, and the clinical features associated with those molecular lesions.

Materials and Methods

Patients

Two cohorts consisting of clinically well-characterized subjects with a clinical diagnosis of NS or features within the RASopathy phenotypic spectrum were included in the study. A first group of patients (N=214) were negative for mutations in genes previously identified to cause NS or a closely related condition (i.e., PTPN11, SOS1, KRAS, NRAS, RIT1, RAF1, BRAF, MAP2K1, SHOC2, and RRAS). In the second cohort (N=135), mutations in a subset of disease genes had been excluded (i.e., PTPN11, SOS1, RAF1, RIT1, KRAS, and SHOC2). Nearly all patients of both cohorts were of European ancestry. Subjects were assessed by experienced clinical geneticists and pediatricians. Clinical assessment included physical, anthropometric, neurologic, and cardiac evaluations, as well as accurate examination of craniofacial features, and ophthalmologic, immunologic, ectodermal, and skeletal defects. DNA samples and clinical data were collected under research protocols approved by an institutional review board, and informed consent for genetic analyses was obtained from all patients.

Mutation Analysis

Genomic DNA was isolated from peripheral blood leukocytes and other tissues, according to standard procedures. The entire CBL coding sequence (NM_005188.3), as well as the exon/intron boundaries and flanking intronic portions, were scanned for mutations by direct sequencing or DHPLC analysis. Sequencing used the ABI BigDye Terminator Sequencing Kit v.1.1 (Applied Biosystems, Foster City, CA) and an ABI 3700 Capillary Array Sequencer or ABI 3500 Genetic Analyzer (Applied Biosystems). DHPLC screening was performed using the 3100 and/or 3500HT Wave DNA Fragment Analysis System (Transgenomic, Omaha, NE), at column temperatures recommended by the Navigator version 1.5.4.23 software (Transgenomic), as previously described [Martinelli et al., 2010]. Amplimers having abnormal elution profiles were reamplified, purified (Qiagen, Hilden, Germany), and sequenced bidirectionally. Homozygous/hemizygous condition for CBL sequence variations was explored by DHPLC analysis using pooled DNAs. Primer pair sequences, and PCR and DHPLC settings are available upon request. Nucleotide numbering of the mutations and exonic disease-unrelated variants reflects cDNA numbering, with 1 corresponding to the A of the ATG translation initiation codon in the reference sequence (NM_005188.3), whereas position of the intronic variants were numbered according to the reference genomic sequence (NG_016808.1).

Mutational screening was also performed on exons 8 and 9 of CBLB (NM_170662.3), and exons 7 and 8 of CBLC (NM_012116.3). Primer sequences are listed in Supp. Table S1.

When available, parental DNAs were sequenced to establish whether the identified changes in sporadic cases were de novo. Paternity was confirmed using the AmpDESTER ProfilerPlus kit (Applied Biosystems).

Molecular Characterization of Splice-Site Mutations

Total RNA was isolated from circulating leukocytes of case 12520-1 by RNasy Mini Kit (Qiagen). Reverse transcription was performed using the SuperScriptIII first strand kit (Invitrogen, Carlsbad, CA), following the manufacturer’s instructions. RT-PCR was carried out using primers designed to amplify exons 7–9 of CBL. Primer sequences are listed in Supp. Table S2. The length of each transcript was determined by cloning purified RT-PCR products in a pCR 2.1 TOPO vector (Qiagen). Individual Escherichia coli colonies were amplified, and amplicons were separated by electrophoresis on 2% agarose gel, and sequenced.

RNA was not available for case 827-10. To characterize the effect of the c.1096-1G>T mutation on mRNA processing, we generated wild-type and mutant minigenes, using the exon trapping vector pSPL3 (Life Technologies, Monza, Italy). A genomic DNA fragment encompassing exons 7–9 of CBL (Chr11:119147899–119149924)
was amplified by using Intron 6_Fw and Intron 9_Rv primers including the XhoI and NheI restriction sites, respectively (Supp. Table S2). PCR amplification was performed using the Platinum Taq DNA Polymerase (Invitrogen). The resulting 2,025-bp-length PCR product was purified, digested, and cloned into the pSPL3 vector. The correct sequence of the fragment of interest was confirmed by direct sequencing. COS1 cells were transiently transfected with 4 μg of wild-type or mutant constructs using Fugene 6 (Promega, Milan, Italy). Twenty-four hours after transfection, cells were harvested, total RNA was extracted, and reverse transcription was performed as described above. The resulting cDNAs were amplified using pSPL3-specific primers (SD6 and SA2) and primers to amplify CBL exons 7–9. PCR conditions are available upon request.

**Functional Studies**

The c.705T>A nucleotide substitution was introduced in the N-terminally hemagglutinin (HA)-tagged human CBL cDNA cloned in the pALTER-MAX vector (Promega) by site-directed mutagenesis (QuikChange Kit; Stratagene, La Jolla, CA). COS1 cells were transiently transfected with wild-type or mutant CBL (4 μg), by using Fugene 6 (Promega). Twenty-four hours post-transfection, cells were serum-starved (16 hr) and then stimulated with EGF (Life Technologies) (100 ng/ml, 30 min). ERK and AKT phosphorylations assays were performed in cells transiently expressing the p.Tyr235*, p.Arg420Gln, and wild-type CBL proteins, as previously described [Martinelli et al., 2010, 2012]. Protein phosphorylation status (pERK1/2 #9106 and pAKT #9271S; Cell Signaling, Danvers, MA) was evaluated basally or after EGF stimulation. ERK1/2 (Cell Signaling, #9102), AKT (Cell Signaling, #9272), CBL (anti-HA antibody; H3663; Sigma–Aldrich, St. Louis, MO) and β-tubulin (T4026; Sigma–Aldrich) expression levels were measured. For time course analysis of CBL stability, 24 hr after transfection, COS1 cells were treated with cycloheximide (Sigma–Aldrich) at 100 μg/ml to prevent protein synthesis, at the indicated times.

**Statistical Analysis**

P values for genotype–phenotype correlation analyses were calculated using two-tailed Fisher’s exact test. The significance threshold was set at P = 0.05. Exact confidence intervals of proportions (at 95% level) were calculated based on binomial distribution.

**Results**

**Mutation Analysis and Functional Characterization of Mutations**

Three-hundred and forty-nine subjects with clinical diagnosis of NS or having features suggestive of this disorder or within the RASopathy phenotypic spectrum, and without mutations in previously identified NS disease genes were included in the study. Mutation analysis allowed the identification of heterozygosity for a CBL mutation in five unrelated individuals (Fig. 1A; Table 1) with a variable phenotype overlapping NS only in part (Supp. Table S3) (NS_EuroNet database, https://nseuronet.com/php/index.php). The c.1111T>C (case 10-0746) and c.1104_1112del (case 13M1230) mutations (p.Tyr371His and p.Glu369Lys, respectively), as well as the c.1096-4_1096-1delAAAG (case 12520–1) and c.1096-1G>T (case 827-10) splice-site variants were identified in four sporadic cases. Among these, parental DNA was available for three patients, and sequencing of the relevant exon demonstrated the de novo origin of mutations (Table 1). In cases for which DNA from other tissues was available, the CBL lesion was documented in oral mucosal epithelial (c.1111T>C) or muscle (c.1096-4_1096-1delAAAG) cells, which excluded a somatic event restricted to hematopoietic cells (Supp. Fig. S1). The c.705T>A substitution in exon 4 was documented in a child (case 05-0562) whose mother and brother showed features within the RASopathy spectrum. DNA specimens from these family members were not available to analyze cosegregation of the mutation with disease. This change, however, was not observed in more than 400 population-matched unaffected individuals examined by DHPLC analysis and direct sequencing, indicating that this variant does not represent a common disease-unrelated polymorphism occurring in the population. Consistently, this variant had not been reported in public databases (dbSNP138, ExAC, and 1000 genomes). A full list of the disease-unrelated changes, including silent substitutions and intronic variants close to the intron/exon boundaries, is reported in Supp. Table S4. Given the close homology and functional relationship occurring among the three members of the CBL family (Swaminnathan and Tsygankov, 2006), the exons of CBLB (MIM #604491) and CBLC (MIM #608453) encompassing the sequence encoding the RING finger domain and the LHR, which represent the mutational hotspot regions for CBL (see below), were analyzed in a subset of 96 subjects within the first cohort. Mutation scanning failed in identifying any disease-causing lesion in these genes, indicating that CBLB and CBLC are not commonly mutated in RASopathies.

The p.Tyr371His and p.Glu369_Tyr371del mutations involve residues evolutionarily conserved in CBL orthologs and paralogs located within the LHR, a region frequently affected by somatic and germline CBL lesions (Fig. 1B). Homozygous defects involving Tyr371 and Lys372 are the most common event among CBL mutation-positive individuals with nonsyndromic JMML [Loh et al., 2009; Muramatsu et al., 2010; Niemeyer et al., 2010]. Leukemia-associated mutations affecting Tyr371 were previously shown to abolish RTKs ubiquitination by impairing E3 activity [Levkowitz et al., 1999; Thien et al., 2001; Sanada et al., 2009; Niemeyer et al., 2010], promote cytokine-independent growth and constitutive activation of downstream signaling pathways (i.e., RAS-MAPK and PI3K-AKT) [Niemeyer et al., 2010], and confer oncogenic properties [Kassenbrock and Anderson, 2004; Sanada et al., 2009]. Likewise, p.Glu369_Tyr371del, which had been reported as a somatic event associated with 11q-UPD in a subject with chronic myelomonocytic leukemia (CML) [Sanada et al., 2009], is expected to be functionally equivalent to mutations affecting Tyr371.

Both the c.1096-4_1096-1delAAAG and c.1096-1G>T lesions affect the CBL splice acceptor site for exon 8. Splice-site mutations affecting introns 7 and 8 account for approximately 10% of CBL defects underlying myeloid malignancies [Kales et al., 2010], and are predicted to encode a protein that lacks essential regions of the LHR and/or the RING finger domain. RT-PCR analysis performed on cDNA obtained from total RNA extracted from circulating leukocytes of patient 12520-1 documented aberrant processing of the transcript. Besides the wild-type allele, three smaller products resulting from partial or complete deletion of exon 8 were identified (Fig. 2A and C; Supp. Fig. S2). Interestingly, products 1A and 1B resulted from the use of two alternative splice acceptor sites within exon 8 (c.1118_1119 or c.1166_1167), leading to deletion of the first 8- or 24-hr residues encoded by this exon. The c.1096-1G>T transversion found in case 827-10 had not been previously described, but a different change at the same position (c.1096-1G>C) had been reported [Abbas et al., 2008; Grand et al.,...
Figure 1. RASopathy-associated germline CBL mutations. A: Electropherograms showing the heterozygous CBL mutations identified in the present study. Nucleotide deletions are shown in open boxes. B: Location of germline disease-associated lesions is shown above the CBL domain structure scheme (splice-site changes are shown as green-filled peaks). Forty-four germline CBL mutations have been identified to date. Amino acid changes at codons 371 and 367 have been reported in 18 and four unrelated cases, respectively; mutations at codons 390, 418, and 420, and the c.1228-2A>G splice-site change, have been found in two cases; all the other substitutions were described once. The N-terminal tyrosine kinase binding domain (TKB) (red), linker helix region (LHR) (light gray), RING-finger domain (RING) (orange), and ubiquitin-associated and leucine zipper domain (UBA/LZ) (dark gray), are shown. The distribution of somatic CBL missense (blue-filled peaks), nonsense (red-filled peaks), and splice-site (green-filled peaks) mutations occurring in leukemia is shown below the cartoon (Cosmic database). C: Facial features of two affected subjects carrying the c.705T>A (p.Tyr235*) and the c.1111T>C (p.Tyr371His) mutations.
<table>
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<th>Number of cases</th>
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<th>Exon</th>
<th>Predicted amino acid change</th>
<th>Domain</th>
<th>Zygosity hematopoietic cells</th>
<th>Zygosity non-hematopoietic cells</th>
<th>Origin of the mutation</th>
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<td>Present study</td>
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<td>Present study</td>
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<td>Familial</td>
<td>Strullu et al. (2013)</td>
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*Position referred to the A of the ATG translation initiation codon in the reference cDNA sequence (NM_005188.3).

bA aberrant mRNA processing was demonstrated, leading to a catalytically inactive protein missing a portion of both the RING domain and the linker helix region connecting it to the TKB domain (mutations affecting intron 7), or the RING domain alone (mutation affecting intron 8).

cA missense mutation was found in an homozygous state in three independent teratoma samples. Nonhematopoietic cells were not available in two subjects carrying this lesion.

dThis mutation occurred as a de novo event in eight subjects, whereas it was inherited in six cases.

LHR, linker helix region connecting the RING finger domain to the TKB domain; Linker2, stretch connecting the RING finger domain to the proline-rich domain; NA, data not available; RING, RING finger domain; TKB, N-terminal tyrosine kinase-binding domain.

2009; Loh et al., 2009; Niemeyer et al., 2010]. Since RNA was not available for this patient, a minigene assay was performed to explore the consequences of the c.1096-1G>T variant on mRNA processing [Cooper, 2005]. COS1 cells were transiently transfected with the pSPL3 constructs containing either the wild-type or mutant CBL genomic region encompassing exon 8. Sequencing of the processed transcript RT-PCR products revealed aberrant splicing of the mutant allele, resulting in loss of the entire exon 8 (Fig. 2B and C; Supp. Fig. S2).

The c.705T>A transversion found in case 05-0562 is predicted to cause premature termination of the protein at codon 235, within the TKB domain, possibly generating a n-Cbl-like protein lacking E3 activity [Langdon et al., 1989; Blake et al., 1991]. RNA was not available to evaluate the extent of reduction in the level of mRNA of the transcribed mutant allele due to nonsense-mediated decay. To explore the effects of this change on protein expression and stability in vitro, and evaluate its impact on signal flow through the RAS-MAPK and PI3K-AKT cascades, we transiently expressed HA-tagged CBLWT, CBLTyr235*, and CBLArg420Gln proteins, the latter included as representative of CBL mutants acting in a dominant-negative manner [Sargin et al., 2007; Dunbar et al., 2008; Martineili et al., 2010], in COS1 cells. Western blot analysis showed a dramatically reduced expression of the CBL Tyr235* mutant compared with the wild-type protein (Fig. 3A), which was due to quick protein degradation (half-life ≈2 hr) as documented by cycloheximide treatment experiments (Fig. 3B). Cells expressing the CBLTyr235* mutant, however, showed enhanced constitutive and EGF-induced ERK and AKT phosphorylation (Fig. 3C), suggesting a dominant-negative effect on wild-type CBL, possibly uncoupling cell-surface receptor binding to E3 ligase activity.

Clinical Features of CBL Mutation-Positive Subjects

Detailed clinical information was obtained for the five subjects harboring the germline CBL mutations (Supp. Table S3), and pic-
Consequences of the c.1096-4_1096-1delAAAG and c.1096-1G>T splice-site mutations [B58, performance percentile), weight 64, verbal percentile). When he was admitted for genetic re-evaluation at the age of 3 years and 2 months, he was noted to have asymmetric thorax and relatively dark skin pigmentation.

The boy heterozygous for the c.1096-4_1096-1delAAAG splice-site change had an unremarkable family history. At birth, she was hydropic and profoundly hypotonic with no respiratory effort, and required immediate resuscitation and intubation. She exhibited severe respiratory acidosis, marked bilateral pleural effusions, and chylothorax, and was found to be at grade 2 of Sarnat’s hypoxic-ischemic encephalopathy scale. Her physical examination revealed diffuse cyanosis, anasarca, mild strabismus, redundant skin on the neck, shield chest with widely spaced nipples, extremities joint limitations, left hand single palmar crease, hammer toes, high arched feet, and diffuse petechiae on face and upper body. Cardiac evaluation revealed pulmonary valve stenosis and biventricular hypertrophy with severe left outflow obstruction. Neurological examination demonstrated severe hypotonia with limited spontaneous activity. Brain ultrasonow showed increased white matter echogenicity and mildly underdeveloped sulci in the brain parenchyma. She also exhibited hepatosplenomegaly and bilateral hydrenephrosis and hydroureretes. Blood count showed persistent leukocytosis, monocytosis, and thrombocytopenia, whereas bone marrow aspirate presented elevated blast count with no evidence of chromosome rearrangements. On liver biopsy, there was prominent extramedullary hematopoiesis, supporting a diagnosis of JMML. Head circumference and linear growth decelerated quickly, settling below the 3rd percentile. At the age of 5 months, neurodevelopmental assessments demonstrated evidence of global developmental delay with poor interaction with the environment and delayed motor skills. Severely bradycardic and hypotensive, she died at the age of 6 months. Autopsy revealed diffuse edema, bilateral pulmonary effusions, extensive multifocal fibrosis, and perivascular infiltrates of nucleated erythroid and myeloid precursor cells, suggestive of extramedullary hematopoiesis.

The boy heterozygous for the c.1096-1G>T splice-site variant had a history of prenatal pleural effusions/hydrops. Clinical details had recently been reported in a separate study focused on prenatal findings associated with germline CBL mutations [Bülow et al., 2015]. Major postnatal features included severe feeding difficulties, failure to thrive, short stature, hypotonia, and psychomotor retardation. Splenomegaly and easy bruising without any evidence of a hematologic neoplasia persisted up to the age of 11 years, when he was last examined. Cardiac evaluation revealed mild pulmonary valve stenosis. He also showed a broad chest with mild pectus excavatum and carinatum, and hyperpigmentation of the legs. Blood count showed persistent leukocytosis, monocytosis, and thrombocytopenia, whereas bone marrow aspirate presented elevated blast count with no evidence of chromosome rearrangements. On liver biopsy, there was prominent extramedullary hematopoiesis, supporting a diagnosis of JMML. Head circumference and linear growth decelerated quickly, settling below the 3rd percentile. At the age of 5 months, neurodevelopmental assessments demonstrated evidence of global developmental delay with poor interaction with the environment and delayed motor skills. Severely bradycardic and hypotensive, she died at the age of 6 months. Autopsy revealed diffuse edema, bilateral pulmonary effusions, extensive multifocal fibrosis, and perivascular infiltrates of nucleated erythroid and myeloid precursor cells, suggestive of extramedullary hematopoiesis.

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The boy carrying the de novo c.1104_1112del mutation exhibited congenital left-sided cataract that had been diagnosed during the neonatal period. Echocardiography revealed mild pulmonary valve stenosis requiring no specific treatment. He was operated for inguinal hernia and undescended testis at the age of 18 months. No hematologic abnormality was ever noted. Psychomotor development was significantly delayed (walking at 30 months). At the age of 35 months, his height was 91.5 cm (10th percentile), weight was 14.1 kg (50th percentile), and head circumference was 48 cm (3rd percentile). When he was admitted for genetic re-evaluation at the age of 3 years and 2 months, he was noted to have asymmetric thorax and relatively dark skin pigmentation.

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A: Expression levels of exogenous CBL in COS1 cells transiently expressing wild-type CBL, and the CBL<sup>Arg420Gln</sup> and CBL<sup>Tyr235+</sup> mutants. Whole cell extracts were probed with anti-HA and anti-β-tubulin antibodies.

B: Time course analysis of CBL stability after cycloheximide treatment. Twenty-four hours following transfection, COS1 cells were treated with cycloheximide at 100 μg/ml to prevent protein synthesis, at the indicated times, and probed with anti-HA and anti-β-tubulin antibodies. Blot with the truncated mutant (right panel) was exposed four times longer than that displaying the wild-type and p.Arg420Gln proteins (left panel) to appreciate the expression levels of the former.

C: Determination of ERK and AKT phosphorylation levels in transiently transfected COS1 cells. Protein phosphorylation status (pERK1/2 and pAKT) was evaluated basally or following EGF stimulation (100 ng/ml, 30 min). Total ERK and AKT levels in cell lysates are shown for equal protein expression and loading. Representative blots of three performed experiments are shown.

**Clinical Spectrum of CBL Mutations and Genotype–Phenotype Correlation Analyses**

A review of the literature allowed us to collect extensive clinical data for 13 subjects carrying germline CBL mutations, whereas incomplete information was available for 18 additional individuals originally studied on the basis of JMML/AML occurrence (Supp. Table S3). Overall, analysis of the available clinical records confirmed previous observations from our group and others indicating that CBL mutations are associated with a variable phenotype resembling, in part, NS, and confer predisposition to JMML. Notably, while a particularly severe phenotype was observed in a small subset of patients, clinical features appeared quite subtle in the majority of cases. We noticed a lower prevalence of individuals exhibiting reduced growth (stature below the 3<sup>rd</sup> percentile) (11/36 vs. 84/115, \( P < 0.0001 \), Fisher’s exact test), cryptorchidism (5/18 vs. 64/83, \( P < 0.0002 \)), and cardiac defects (12/36 vs. 132/151, \( P < 0.0001 \)), compared with what observed in the NS general population [Sarkozy et al., 2009]. Pulmonary valve stenosis and HCM were detected in 17% (\( P < 0.0001 \)) and 8% of cases, respectively. Craniofacial features were always present but generally mild. Ectodermal and skeletal involvement was frequently observed, with thin/sparse hair, café-au-lait spots, and thorax anomalies being the most recurrent features. Five out of 17 cases reported by Niemeyer et al. (2010) and one additional patient [Strullu et al., 2013] exhibited juvenile xanthogranuloma. Interestingly, CBL mutation-positive subjects displayed a relatively high frequency of neurological features, including psychomotor delay (65%), cognitive delay (35%), and muscular hypotonia (50%). Head MRI documented delayed myelination, abnormal corpus callosum, and cerebellar vermis hypoplasia in two unrelated individuals, whereas Arnold–Chiari malformation type I, mild left cerebral atrophy, widened cisterna magna, and moyamoya disease were reported in one patient each. Recurrent complications also included ocular anomalies (22%) and hearing loss (17%).

**Discussion**

CBL is a widely expressed E3 ubiquitin ligase that negatively regulates intracellular signaling downstream of RTKs [Swaminathan and Tsygankov, 2006; Dikic and Schmidt, 2007]. Germline CBL mutations have been established to cause a previously unrecognized RASopathy with variable phenotype [Martinelli et al., 2010; Niemeyer et al., 2010; Pérez et al., 2010]. In this work, we provided new data on the molecular spectrum of germline CBL mutations.
and more comprehensively assessed the clinical features associated with CBL lesions.

Since the discovery of CBL as a RASopathy gene, 44 germline CBL mutations have been identified. Combined data indicate that these lesions are predominantly missense changes (82.4%; 95% CI, 69.6%–95.2%) affecting residues located within the RING finger domain or the LHR. Among those lesions, approximately 50% involves Tyr371, with the p.Tyr371His amino acid substitution being the most recurrent change. Tyr371 plays a crucial role in the activation of CBL E3 ligase activity [Dou et al., 2012]. The available crystal structure data are consistent with a model of CBL functional regulation in which, in the absence of the substrate, the enzyme adopts an autoinhibited conformation with the TKB domain binding to the RING finger domain and masking the E2-binding sites. Following Tyr371 phosphorylation, the LHR undergoes a conformational change that abrogates autoinhibition and brings the RING domain and E2 nearby the substrate-binding site, thus promoting E3 activity. Extensive studies demonstrated that E3 activation is required for RTKs ubiquitination [Levkowitz et al., 1999; Thien et al., 2001; Sanada et al., 2009; Niemeyer et al., 2010]. Consistent with that, mutations affecting Tyr371 was shown to abrogate the ability of CBL to ubiquitinate FLT3, KIT, and EGFR [Sanada et al., 2009; Niemeyer et al., 2010].

Heterozygous variants affecting splicing of CBL have been observed in a significant fraction of cases (11.8%; 95% CI, 0.9%–22.6%), the majority resulting in loss of the sequence encoding the most recurrent change. Tyr371, in the present cohort, case 12520-1 carrying the c.1096_4,1096_1delAAAG mutation, which disrupts the splice acceptor site for exon 8, exhibited a severe RASopathy phenotype with JMML. Interestingly, we failed to identify loss of the wild-type allele in leukemic cells from this subject. This finding mirrored available data indicating that somatically acquired CBL deletions arising from aberrant splicing are frequently heterozygous, whereas the vast majority of missense mutations are homozygous as a consequence of 11q isodisomy [Kales et al., 2010; Strullu et al., 2013]. In line with this evidence, the splice-site mutations detected in MOLM-13 and MOLM-14 cell lines and NUP98-HOXD13 transgenic mice were also heterozygous [Caligiuri et al., 2007; Abbas et al., 2008; Slape et al., 2008; Reindl et al., 2009]. These findings suggest that in-frame deletions of the LHR/RING domains of CBL might confer stronger transforming properties than missense mutations. Further studies are required to test this hypothesis.

One small in-frame indel and a nonsense mutation occurring in two patients were also identified. While the former, leading to deletion of three highly conserved residues including Tyr371, had previously been described in leukemia [Sanada et al., 2009], the latter is a novel change causing premature termination of the protein at residue 235, within the calcium-binding EF hand of the TKB domain. This motif plays a key role in maintaining the TKB structure, allowing proper binding of the ligase to protein tyrosine kinases [Lusher et al., 1999; Meng et al., 1999]. Truncating mutations affecting the TKB domain of CBL rarely occur as somatic events in cancer (COSMIC database, http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). In particular, heterozygous nonsense mutations at codons Gln275 and Arg443 were observed in a lung carcinoma sample and a CMML subject carrying a concomitant p.Cys384Try amino acid change, respectively [Sanada et al., 2009; Imielski et al., 2012]. These transcripts coding for truncated proteins are expected to be recognized and degraded via nonsense-mediated mRNA decay (NMD), an evolutionary conserved mechanism used by eukaryotes to prevent the production of aberrant proteins with deleterious gain-of-function or dominant-negative effects [Frischmeyer and Dietz, 1999], suggesting haploinsufficiency as the underlying molecular mechanism. Accumulating data, however, document high variability in NMD efficiency [Miller and Pearce, 2014], suggesting that a consistent fraction of these nonsense transcripts might generate v-Cbl-like proteins with dominant-negative function. v-Cbl, the transforming gene of the Cas NS-1 retrovirus, is a truncated 357 residue-long CBL protein able to induce B-cell lymphomas and myeloid leukemia in mice [Langdon et al., 1989; Blake et al., 1991]. This protein functions in a dominant-negative manner by uncoupling CBL binding to activated RTKs from their ubiquitination and degradation. Consistently, our in vitro functional data documented enhanced ERK and AKT phosphorylation in cells expressing the p.Tyr235* mutant.

Extensive studies demonstrate that germline-transmitted and somatically acquired mutations in RASopathy genes rarely overlap [Tartaglia et al., 2011], PTPN11 being the archetypal example [Tartaglia et al., 2006]. In that case, NS-causing mutations confer milder gain-of-function effects than the cancer-associated lesions do, with the former being sufficient to perturb biological processes that are strictly controlled by SHP2, but inadequate to deregulate significantly processes that are less strictly controlled by the phosphatase. Similarly, mutations in NRAS, KRAS, and BRAF underlying NS and cardiofaciocutaneous syndrome (CFCs; MIM #115150), as well as those involving HRAS in Costello syndrome (CS; MIM #218040), rarely occur as oncogenic defects [Aoki et al., 2008; Tartaglia et al., 2011]. Conversely, the molecular spectra of germline and somatic CBL mutations largely overlap, suggesting that these lesions have generally milder consequences on development than cancer-associated mutations in most RASopathy genes. Of note, however, missense mutations involving Tyr371 have been identified rarely in subjects with CBL mutation-associated syndrome without JMML (~10% of cases), whereas they represent the most common lesions among individuals with CBL mutation-positive syndromic JMML (~50% of cases). This observation indicates that patients carrying a germline defect altering codon 371 are at risk for developing JMML. Consistent with this, codon 371 represents a “hot spot” for somatic CBL mutations in isolated JMML, whereas it is rarely mutated in acute leukemias [Kales et al., 2010].

The present study is in line with our previous estimate of CBL mutation prevalence, indicating that defects in this gene account for ~1% of subjects with clinical features resembling NS, even though they might be more common among individuals with features within the RASopathy phenotypic spectrum. Although the clinical spectrum associated with CBL mutations is wide, genotype-phenotype correlations seem to exist. Specifically, CBL mutation-positive patients exhibit a relatively lower prevalence of cardiac defects (33%) compared with the general NS population, for which an estimate of up to 80% has been reported [Burch et al., 1993; Marino et al., 1999; Shaw et al., 2007; Sarkozy et al., 2009]. Of note, CHDs were observed in all subjects carrying the p.Glu367Pro amino acid change (N = 4), whereas only one out of 12 patients with a missense mutation at codon 371, for which clinical data were available, had a cardiac defect (HCM). Short stature is a common feature in NS, although adult height is not always adversely affected. Clinical data revealed that length/stature is less frequently below the 3rd percentile (31%) in cases with mutated CBL compared with PTPN11 mutation-positive NS subjects (93%) [Roberts et al., 2013], similar to what reported in individuals with SOS1 mutations [Tartaglia et al., 2007; Lepri et al., 2011]. Finally, CBL mutation-positive patients displayed a high frequency of head MRI findings associated or not with structural brain abnormalities (54%), which are usually rare in NS, but more frequently occur in other RASopathies, such as CFCs [Roberts et al., 2006]. Among these, case 827-10 showed delayed brain myelination, a feature that
was observed in one subject from our previous cohort [Martinelli et al., 2010]. Similar abnormalities represent a relatively common feature in CFCS patients with a BRAF mutation [Yoon et al., 2007; Aizaki et al., 2011]. Of note, conditional ablation of Braf in mouse neuralgial precursor cells was shown to result in defective myelination and oligodendrocyte differentiation [Galabova-Kovacs et al., 2008]. Similarly, loss of Shp2 in the oligodendrocyte lineage, as well as transgenic expression of a NS-associated Shp2 gain-of-function allele, results in severe abnormal myelination phenotypes [Ehrman et al., 2014]. Hence, the myelination defect observed in some individuals with a CBL mutation adds support to the impact of dysregulated RAS signaling on glial cell development. Of note, patient 10-0746 and case HD316 from our previous cohort [Martinelli et al., 2010] showed cerebellar vermis hypoplasia, an unreported feature in RASopathies. Besides these atypical findings, additional brain anomalies (e.g., Arnold–Chiari malformation type I, hypoplastic corpus callosum, mild brain atrophy, and cerebrovascular defects) occur more frequently in NS and related disorders [Schon et al., 1992; Ganesan and Kirkham, 1997; Galarza et al., 2010; Aizaki et al., 2011; Keh et al., 2013; Okamoto et al., 2014].

Overall, the present analysis indicates that patients heterozygous for a germline CBL mutation exhibit a wide phenotypic variability partially overlapping NS. Besides the occurrence of a subtle phenotype in a large proportion of subjects, clinical features are particularly severe in a small number of cases. Although available data suggest possible genotype–phenotype correlations, based on the increasing evidence documenting co-occurrence of mutations in functionally related genes [Nystrom et al., 2009; Tang et al., 2009; Keh et al., 2013; Okamoto et al., 2014].

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