Table 1 Characteristic features of different types of CDA

CDA type	I	II	III familial
Inheritance	Autosomal recessive	Autosomal recessive	Autosomal dominant
Case reported	~150	>300	3 families
Incidence	<1/100000/year	<1/100000/year	Very rare
Blood smear	Anisopoikilocytosis with basophilic stippling, occasional circulating mature erythroblast	Anisopoikilocytosis with basophilic stippling, occasional circulating mature erythroblast	Anisopoikilocytosis with basophilic stippling
Bone marrow erythroblasts (light microscopy)	Abnormal chromatin structure, binucleated, chromatin bridges	Normoblastic, multinuclearity of mature erythroblasts	Giant multinucleated erythroblasts
Bone marrow erythroblasts (electron microscopy)	Spongy "Swiss-cheese" appearance of heterochromatin	Peripheral double membranes	Nonspecific, intranuclear clefts, karyorrhexis
Age at presentation	In utero (hydrops fetalis), neonatal, childhood or early adulthood	Most are diagnosed from age 5 to 30 years (average 18–20)	N/A
Symptoms and signs	Anemia, iron overload, splenomegaly and hepatomegaly, extramedullary hemopoiesis	Anemia and jaundice, gallstones, splenomegaly (usually by adulthood), iron overload, paravertebral hematopoiesis, aplastic crisis	Mild anemia, no iron overload
Associated dysmorphism	Skelton, others	Rare	B cells, retina
Hemoglobin (g/dl)	6.5–11.5 (mean = 9.5)	9–12 (mean = 11)	8–14 (mean = 12)
MCV	↑↑ (70% cases)	Normal/minimal ↑	Normal/minimal ↑
RDW	↑↑↑	↑↑	↑↑
Reticulocytes	Suboptimal response		
Total bilirubin (mg/dl)	↑ (indirect)	↑ (2–8)	Normal/minimal ↑
LDH	↑	↑	↑↑↑
Ferritin (μg/l)	↑ (in 60% = 1000–1500)	↑↑ (>50% >1000 by age 50 years)	Normal
Ham test	Negative	Usually positive	Negative
Anti-i antigen hemagglutination	Normal–strong	Strong	Normal–strong
Serum thymidine kinase	↑↑↑	↑↑	↑↑↑
Altered red cell proteins	Low levels of protein 4.1 Impaired globin synthesis	Unusual appearance of band 3 protein Reticular endoplasmic proteins	Normal
Hemosiderinuria	N/A	N/A	+++
Glycosylation	Some abnormality	Markedly abnormal	Some abnormality
Gene	*CDAN1* (Codanin-1) 15q 15.1.3	*CDAN2* 20q 11.2	*CDAN3* 15q 21–25
Therapy	Interferon-α, iron depletion	Splenectomy, iron depletion	None

with increased direct bilirubinemia and transaminases, and persistent pulmonary hypertension of the newborn [20]. Clinical appearance and basic laboratory data are similar to CDA type II with some exceptions. Most cases show lifelong anemia with hemoglobin concentration between 8 and 11 g/dl. Red blood transfusions are often required for newborns and infants, but are only occasionally necessary later in life [4, 19, 20, 22]. Anemia is usually macrocytic with MCVs between 100 and 120 fl. Symptoms can be highly variable, and mild cases may remain undetected. Physical abnormalities are more frequently observed than in CDA type II. These patients show skeletal malformations, particularly syndactylism of hands and feet, absence of nails or additional toes. Short stature may be the result of pituitary failure due to unrecognized secondary hemochromatosis. More severe presentations can include gallstones, cholecystitis from chronic hyperbilirubinemia, or extramedullary hemopoietic foci in the parietal and frontal bones of the skull [4, 9, 23]. Although iron overloading is rarely the primary symptom, it seems to be present in most patients after childhood. Iron overload is not limited to patients receiving frequent transfusion, because the highly ineffective erythropoiesis results in decreased hepcidin levels, and thus increased iron absorption [24]. Liver cirrhosis, increased skin pigmentation and endocrine dysfunction as a result of iron overload

have been reported in occasional patients [25, 26]. Levels of ferritin increase with age if chelation therapy is not initiated, but tend to be in the range of 500–1500 μg/l in adulthood [19, 22–26] Associations of CDA type I with mutations in the HFE hemochromatosis gene have been described, but do not seem to increase the severity of loading [22, 23].

Serologically, the red cells give a negative result with the acidified serum lysis test and usually show normal agglutinability with anti-i [27]. In the peripheral blood smear, large poikilocytes and elliptocytes are similar to changes seen in megaloblastic anemia. Macrocytes can be observed even in cases in which the MCV is within the normal range. Basophilic stippled cells are present and Cabot rings or Howell–Jolly bodies may be seen in some cases even before splenectomy. In some cases, late normoblasts circulate in the peripheral blood. Light microscopy of the bone marrow demonstrates erythroid hyperplasia with abnormal precursors showing a megaloblastic appearance. There are also occasional tri- and tetranucleate cells. The diagnostic finding in the bone marrow is the presence of internuclear chromatin bridges between some nearly completely separated erythroblasts. This characteristic dysplasia only affects a small percentage of the erythroblasts; a large number of erythroblasts should be assessed when the diagnosis of CDA type I is being considered. With the exception of a few cases of erythroleukemia, internuclear chromatin bridges are almost specific for CDA type I, in contrast to the frequently observed cytoplasmic threads between two erythroblasts in the smear [28]. Electron microscopy of the bone marrow erythroblast shows particularly specific alterations. The abnormalities become distinct with progressing maturation. As much as 40–60% of the intermediate and late erythroblasts show a characteristic heterochromatin pattern. The heterochromatin is abnormally electron dense with a spongy appearance that has been described as Swiss cheese like. The nuclear membrane is frequently invaginated into the nucleus and contains cytoplasmic organelles [26, 28].

In some cases of CDA type I, there are increased amounts of HbA2 and there may also be an increased α/non-α globin chain synthesis ratio. The cause of the unbalanced globin chain synthesis is not known [25, 29].

The genes responsible for CDA type I were mapped to chromosome 15q15.1–15.3 in four consanguineous families of Israeli Bedouins in 1998 [30]. This gene has been identified and designated *CDAN1* in 2002 [5]. This large 15-kb gene has 28 exons and encodes the protein codanin-1 containing 1227 amino acids. Northern blot analysis revealed that the gene is ubiquitously expressed. It is highly conserved evolutionally at least down to fish, and has no orthologs within the human genome, suggesting a nonredundant function. The *CDAN1* gene is mutated in 88% of CDA type I patients, and more than 30 unique mutations have been identified so far. No patients have been found to be homozygous for a null-type mutation, suggesting that the complete absence of functional codanin-1 may be lethal [31, 32]. Recently, codanin-1 was found to be localized to the heterochromatin in interphase cells and was transcriptionally regulated by E2F1, a member of a transcription factor family involved in cell cycling [33].

Treatment for CDA is mostly supportive and targeted to prevent the consequences of anemia and iron overload. Interferon-α was used for the treatment of hepatitis C infection in a patient who had CDA type I. Following interferon therapy, progressive and impressive correction of the hemoglobin levels ware observed. Furthermore, most bone marrow erythroblasts became morphologically normal even on electron microscopy analysis [11]. This finding has led to the successful administration of IFNα to many other patients who had confirmed CDA type I and has proved to be safe and effective in the long term. Other subtypes of CDA do not respond to INFα [12–15]. Erythropoietin is not effective for CDA type I.

3 CDA type II

This type of CDA is the most frequent form of CDAs and the incidence is about 1 in 100000 births per year. The number of reported cases with CDA type II exceeds 300. CDA type II is also known as hereditary erythroblastic multinuclearity with a positive acidified serum lysis test (HEMPAS). The severity of anemia varies from mild to severe. About 10% of patients require red cell transfusion in infancy and childhood, but rarely thereafter. Splenomegaly and gallstones are common. Co-inheritance of Gilbert syndrome increases the extent of hyperbilirubinemia and the risk of gallstone formation [34]. Splenectomy is effective in the majority of patients [4]. Progressive iron overload is seen even in untransfused patients and liver cirrhosis secondary to iron overload develops in about 20% [10].

Hemoglobin in patients with CDA type II is generally between 8 and 11 g/dl with a normal MCV. Red cell are usually normocytic with moderate to marked anisocytosis, anisochromasia and poikilocytosis (including teardrop-shaped poikilocytes), occasionally basophilic stippling cells and a few circulating erythroblasts. There is normoblastic erythroid hyperplasia in the bone marrow with 10–35% of binucleate and rarely multinucleate late polychromatic erythroblasts. Electron microscope shows stretches of double membrane parallel to the inner surface of the erythroblast cell membrane that represents excess smooth endoplasmic reticulum containing proteins

normally found in this organelle: calreticulin, glucose-regulated protein (GRP78) and protein disulfide isomerase [10].

Inheritance of CDA type II is autosomal recessive. Genome-wide linkage analysis localized the disease gene (*CDAN2*) to a 5-cM region of chromosome 20q11. 2 [35]; however, the gene responsible for CDA type II was not identified until recently [36]. Finally, the gene responsible for CDA type II was identified by a German group [37]. The study was epoch making because not only did it identify the responsible gene, but also it showed that the gene defects directly led to production of binucleated cells, which is a hallmark of CDA type II. The gene encoding the secretory COPII component *SEC23B* was mutated CDA type II. The mutations were compound heterozygotes in 17 individuals and homozygotes in 16, including 8 in the 5 consanguineous families. Short hairpin RNA (shRNA)-mediated suppression of *SEC23B* expression recapitulates the cytokinesis defect. Knockdown of zebrafish *sec23b* also leads to aberrant erythrocyte development. The secretory pathway in eukaryotic cells is critical for membrane homeostasis, localization of proteins within cells and secretion of extracellular factors. During a budding reaction, cytoplasmic coat proteins (COPs) are assembled on a membrane surface, capture cargo molecules and polymerize into a cage sculpting different-sized cargo vesicles. So far, severe mutations in genes encoding COP II components have been assigned to human genetic disorders. *SAR1B* defects cause chylomicron retention disease, Anderson disease and Marinesco–Sjogren syndrome [38], and *SEC23A* is mutated in cranio-lenticulo-sutural dysplasia [39]. *SEC23B* impairment has been demonstrated as being responsible for CDA type II. The utility of COP II with different cargo selectivity will be elucidated in the future.

Recent study of 42 patients with CDA type II in Italy and France showed correlations between the mutations and various biological parameters. The authors divided patients into two groups: (1) patients with two missense mutations and (2) patients with one nonsense and one missense mutation. Overall, they found 22 mutations in *SEC23B*. Compound heterozygosity for a missense and nonsense mutation tended to produce more severe clinical presentations than homozygosity or compound heterozygosity for two missense mutations [40].

4 CDA type III

CDA type III is the rarest of the three classical types of CDA and both familial and sporadic cases have been reported [41]. Familial cases have been reported in Sweden Argentina and the USA [42, 43]. The largest is a five-generation family living in northern Sweden, whose ancestry can be traced back to the nineteenth century. It contains more than 30 cases inherited in an autosomal dominant manner [44].

In contrast to CDA type I and II, anemia is mild and iron overload seems not to be clinically significant in patients with familial CDA type III [45]. In the Swedish family, there is an increased tendency to develop monoclonal gammopathy and multiple myeloma, although sporadic cases have demonstrated lymphoproliferative disorders [45–47].

In sporadic cases, the inheritance is unclear. First and second-degree relatives are hematologically normal [48]. The clinical features of the sporadic cases are also extremely variable. Hepatosplenomegaly and significant iron overload have been described in some [3].

The only identifying features of CDA type III are those displayed by the bone marrow. Anisopoikilocytosis, basophilic stripping of red cells and some very large red cells may be found in the peripheral blood smear. In families with hemosiderinuria, iron deficiency may be found. In the bone marrow, there are abundant giant erythroid precursors of all developmental stages. These are frequently multinucleated [42, 43, 48, 49]. Ultrastructural analysis shows intranuclear clefts, abnormal nuclear membrane, cytoplasmic autophagosomes, myelin deposits, hemoglobin precipitates or abnormal mitochondria. Giant multinucleate and mononucleate erythroblasts may also be seen in MDS and erythroleukemia [49].

Linkage analysis in one large multiplex family localized the disease gene (*CDAN3*) to chromosome 15q22, distal to the *CDAN1* [41, 44]. Dyserythropoiesis with multinucleated erythroblasts is not specific to CDA type III, and one case with these features (but additional platelet abnormalities) was shown to be attributable to a *GATA-1* mutation that abrogated the interaction between Gata-1 and Fog-1, indicating that defects in certain erythroid-specific pathways can mimic some phenotypic features of CDA type III [50].

5 Epidemiology of CDAs in Japan

There were virtually no data describing the epidemiology of CDAs until recently. The Japanese Society of Pediatric Hematology conducted a national survey for CDA in children in 2006 [51]. Totally, 12 patients with CDA were identified in this retrospective study: 4 with type I, 3 with type II, 2 with type III, and 3 with variant type. Gene mutation analysis was not done in any of these cases. Next, we conducted a national survey with support from the Ministry of Health, Labour and Welfare of Japan in 2009 and identified additional ten cases. Finally, gene mutation analysis was begun for these patients identified through the

surveys in Japan and at present one patient was possible to have mutations in *SEC23B* (Doisaki and Kojima, unpublished data). We expect that epidemiology of CDA in Japan will be clarified in the near future. We believe that molecular analysis of the disease will contribute not only to the precise diagnosis of patients with CDA, but also to elucidation of pathogenesis of the disease.

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CASE REPORT

Bullous exudative retinal detachment due to infiltration of leukemic cells in a child with acute lymphoblastic leukemia

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Abstract Acute lymphoblastic leukemia (ALL) is known to cause several ocular involvements, but exudative retinal detachment is a rare complication. We describe a case report of a 4-year-old boy with T cell ALL who developed bilateral exudative retinal detachment caused by leukemic infiltration in the retinas after achieving hematological remission. Intravenous steroid pulse therapy and local irradiation reversed the condition, but it recurred concurrently with disease progression after a second relapse in the bone marrow. It is suggested that ophthalmic examination is crucial for ALL patients, especially for those whose white blood cell count is very high at onset.

Keywords Acute lymphoblastic leukemia · Children · Retinal detachment · Aspiration of subretinal fluid

1 Introduction

Ocular involvement in ALL is not uncommon. Reddy et al. [1] reported that 49% of ALL patients had retinopathy at initial diagnosis, such as intraretinal hemorrhages, leukemic infiltration, white-centered hemorrhages, central retinal vein occlusions, and vitreous hemorrhages. However, exudative retinal detachment is a rare complication [1–3]. Additionally, ocular complications usually occur when the disease is clinically active, but rarely during hematological remission [3, 4]. Here, we report a patient with T-ALL who developed severe retinal detachment after attaining hematological remission.

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2 Case report

A 4-year-old boy was admitted to our hospital owing to fever and lymphadenopathy. Physical examination revealed mild hepatomegaly 3 cm below the costal margin and multiple systemic lymphadenopathies that were enlarged up to 2 cm. Peripheral blood levels were as follows: hemoglobin 12.6 g/dl, white blood cells 366 × 10^9/l, and platelet count 77 × 10^9/l. A differential blood count revealed 82.4% lymphoblasts. Bone marrow (BM) aspirate showed massive infiltration of lymphoblasts, which were positive for CD2, CD5, CD7, TdT, and cytoplasmic CD3. SIL/TAL1 fusion gene was detected by RT-PCR. Chest X-ray and CT did not show enlargement of the thymus or lymph node and brain CT did not indicate any abnormalities. Therefore, we diagnosed the patient as having T cell ALL.

The patient was treated in accordance with the TCCSG L04-16 high-risk protocol [5]. However, leukemic cells increased in number even after the initiation of oral prednisolone (PSL) treatment and he developed respiratory failure due to hyperleukocytosis and required mechanical ventilation for 5 days. Initial lumbar puncture on day 8 revealed mild pleocytosis (26/μl) with the presence of leukemic blasts. His general status and hematologic findings were improved by induction therapy consisting of PSL, vincristine, L-asparaginase, daunorubicine, and cyclophoshamide (CPA) and he successfully entered hematological remission on day 33. However, he complained of sudden loss of vision on day 49. The

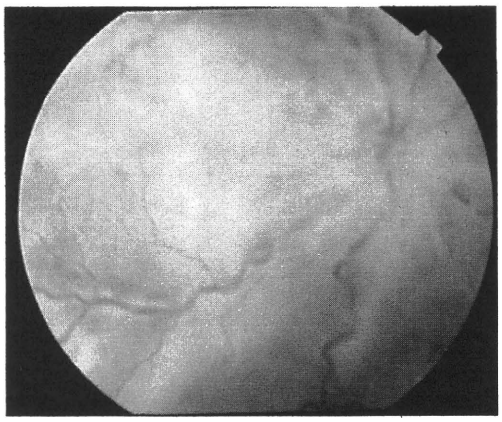

Fig. 1 Fundus photograph of the right eye. Exudative retinal detachment spread from posterior pole to peripheral portion, which was bullous in some parts. Redness and swelling of optic disc, tortuous veins, and intraretinal hemorrhage were also seen

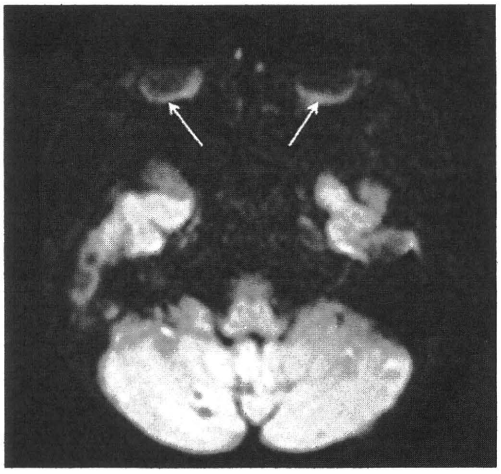

Fig. 2 MRI of brain and orbits. Signal intensity of bilateral retrobulbar areas increased in diffusion imaging

visual acuity was counting fingers in both eyes, but other physical findings, which included skin, testis and central nervous system, were normal. Fundus examination of bilateral eyes showed exudative retinal detachment spreading from the posterior pole including the macula to the peripheral portion, which was bullous in some parts. Redness and swelling of optic disc, tortuous veins, and intraretinal hemorrhage were also seen. There was also perivascular cell infiltration in both eyes (Fig. 1). Ultrasonography demonstrated fluid collection under the retina and an MRI of brain and orbits showed increased signal intensity of bilateral retrobulbar areas in diffusion imaging (Fig. 2). Aspiration of subretinal fluid revealed infiltration of leukemic cells (Fig. 3). At the same time, BM and cerebrospinal fluid (CSF) examination showed no

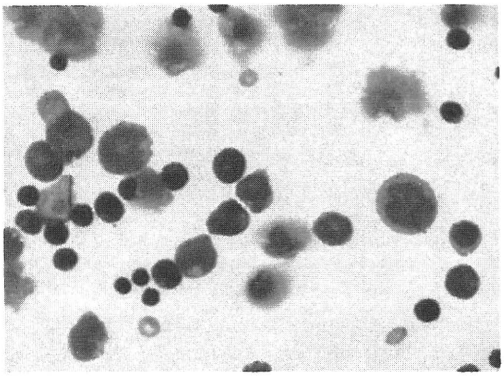

Fig. 3 Infiltration of leukemic cells in the subretinal fluid. Lymphoblasts with cleaved nuclei were seen. A sclerotomy was performed at the temporo-inferior portion at the equator. The choroid was coagulated with diathermy and choroidal puncture was performed with a 27 gauge needle. Yellowish protein-rich subretinal fluid was drained without hemorrhage

evidence of leukemic blasts. Herpes simplex virus, cytomegalovirus, Epstein–Barr virus, and varicella-zoster virus were not detected by PCR of blood, CSF, aqueous humor, and subretinal fluid. Intravenous steroid pulse therapy (30 mg/kg/day × 3 days) and 10 Gy local irradiation dramatically improved his vision up to 20/40 in both eyes. From the results of fundus examination, ultrasonography, and MRI, the subretinal fluid was completely absorbed and the retinas were re-attached. Optic disc swelling also receded. Subsequently, the patient received consolidation chemotherapy, but he developed facial nerve palsy after the third course of therapy and was diagnosed as having a first relapse in BM and the central nervous system. Fundus examination showed recurrence of swelling of the optic disc and perivascular cell infiltration, but no retinal detachment. Brain CT did not show any abnormalities. After he attained a second hematological remission after 2 courses of hyper-CVAD [6], he received allogeneic cord blood transplantation (CBT). The conditioning regimen for CBT was total-body irradiation (TBI), CPA, and etoposide. Cord blood graft containing 6.25×10^7 cells/kg (2.4×10^5 $CD34^+$ cells/kg) was infused without any adverse effects. Graft versus host disease prophylaxis consisted of short-term MTX and cyclosporin A. The post-transplant course was uneventful and neutrophil engraftment was observed on day 19. However, pain of the right upper limb appeared on day 33 and he was diagnosed with second BM relapse on day 37. Lumbar puncture did not show leukemic cell infiltration. He had recurrence of exudative bullous retinal detachment, which caused permanent loss of vision. He died of disease progression 4 months after the second relapse in BM.

3 Discussion

Ocular involvements in ALL are often caused by direct invasion of leukemic cells or hematological abnormalities such as anemia, thrombocytopenia, and hyperviscosity syndrome [7]. Russo et al. [8] reported that orbital or ocular manifestations were noted in 11.5% patients with ALL and more common in patients with high-risk leukemia, who had initial white blood cell count over 500×10^9/l. They also indicated that specific orbital and ocular lesions due to leukemic infiltration were associated with a higher frequency of bone marrow relapse and CNS involvement [8]. With regard to the mechanism of exudative retinal detachment with ALL, leukemic infiltration into the choroid is thought to decrease blood flow in the choriocapillaries and cause ischemia of the overlying retinal pigment epithelium, which disrupts the intercellular tight junction and causes exudative retinal detachment [2]. As a result, incompetence of the outer blood-retinal barrier leads to subretinal accumulation of choroidal fluid [2].

We directly aspirated the subretinal fluid, which contained a large number of leukemic cells. This is the first report that leukemic cell infiltration as a cause of exudative retinal detachment was directly demonstrated by aspiration of subretinal fluid. The retinal invasion of leukemic cells is usually suggested by ultrasonography or by infiltration of leukemic cells into CSF and the resolution of the retinal detachment, which is obtained with systemic chemotherapy [3, 9]. Although aspiration of aqueous humor or vitrectomy is often utilized to detect leukemic cell infiltration, the aspiration of subretinal fluid through the sclera is rarely performed [10]. We considered that direct demonstration of subretinal leukemic cells was helpful to decide therapeutic strategy.

In our case, exudative retinal detachment occurred even though the patient was in hematological remission. It was possible that leukemic cells that infiltrated into the choroid and retina at the time of presentation escaped from the effects of chemotherapy and increased in number rapidly after the completion of remission induction therapy. Chemotherapeutic agents are thought to be difficult to reach inside the eyes owing to the blood–retina barrier. Since the patient's ocular findings deteriorated concurrently with the subsequent disease progression, chemo-resistant clones might survive in the subretinal fluid during chemotherapy.

Since ocular involvements are often caused by leukemic infiltration, it is reasonable to perform systemic chemotherapy first. If systemic chemotherapy fails, ocular radiation at doses greater than 10 Gy is also recommended [3, 4]. Some physicians have claimed that high-dose radiotherapy to the eyes at no less than 30 Gy is needed to avoid a subsequent ocular relapse [11]. In our case, we chose 10 Gy local irradiation and steroid pulse therapy, because the visual impairment rapidly progressed and stem cell transplantation with TBI had been already planned.

Ocular lesions are third most frequent extramedullary location of ALL after CNS and testes and sometimes difficult to manage and could adversely affect the patient's quality of life. Therefore, we suggest that ophthalmic examination, including fundus examination, at diagnosis is crucial for ALL patients, especially for those whose white blood cell count is very high at onset.

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bjh short report

Spectrum of molecular defects in juvenile myelomonocytic leukaemia includes *ASXL1* mutations

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Summary

Mutations in *NF1, PTPN11, NRAS, KRAS* and *CBL* have been reported to play a pathogenetic role in juvenile myelomonocytic leukaemia (JMML), a rare myelodyplastic/myeloproliferative neoplasm occurring in children. Recently, mutations in *ASXL1* were identified in chronic myelomonocytic leukaemia and other myeloid malignancies. We sequenced exon 12 of *ASLX1* in 49 JMML patients, and found 2 novel heterozygous (nonsense and frameshift) mutations, one occurring as a sole lesion, the other was in conjunction with a *PTPN11* mutation. *ASXL1* cooperates with *KDM1A* in transcriptional repression and thereby *ASXL1* mutations may synergize with or mimic other JMML-related mutations.

Keywords: juvenile myelomonocytic leukaemia, *ASXL1*, chronic myelomonocytic leukaemia, *PTPN11*, RAS signalling.

The molecular pathogenesis of juvenile myelomonocytic leukaemia (JMML) includes a number of recurrent mutations and chromosomal aberrations, all resulting in a similar clinical phenotype and the characteristic hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) (Koike & Matsuda, 2008). Constitutional mutations of *NF1*, a GTPase-activating protein that negatively regulates RAS, are associated with the characteristic neurofibromatosis type 1 (NF-1) features and JMML. During leukaemic evolution, heterozygous *NF1* alleles are duplicated through somatic uniparental disomy (UPD) of 17q in 2/3 of mutant cases, while the remaining 1/3 of affected children show compound-heterozygous inactivating *NF1* mutations (Steinemann *et al*, 2010). Other mutations of genes involved in GM-CSF signal transduction, including *RAS* and *PTPN11*, have been reported in 10–20% and 35% of patients with JMML, respectively. Mutations of *PTPN11*, encoding tyrosine phosphatase SHP-2, lead to elevated levels of Ras-GTP, the active form of Ras. Recently, we and others have described ring finger domain mutations of *CBL*, an ubiquitin ligase involved in inactivation of activated phosphothyrosine kinase receptor (TRK)-mediated signals. The presence of *CBL* mutations also explains the laboratory findings of GM-CSF hypersensititvity. Similar to *NF1*, some heterozygous *CBL* mutations can also be constitutional (Loh *et al*, 2009; Muramatsu *et al*, 2009).

In some phenotypic features JMML may resemble chronic myelomonocytic leukaemia (CMML), but cytogenetic and mutational spectra differ between the conditions. While *PTPN11, NRAS* and *KRAS* mutations are not a common finding in CMML, *CBL* mutations have also been found in both CMML and JMML. Based on the identification of

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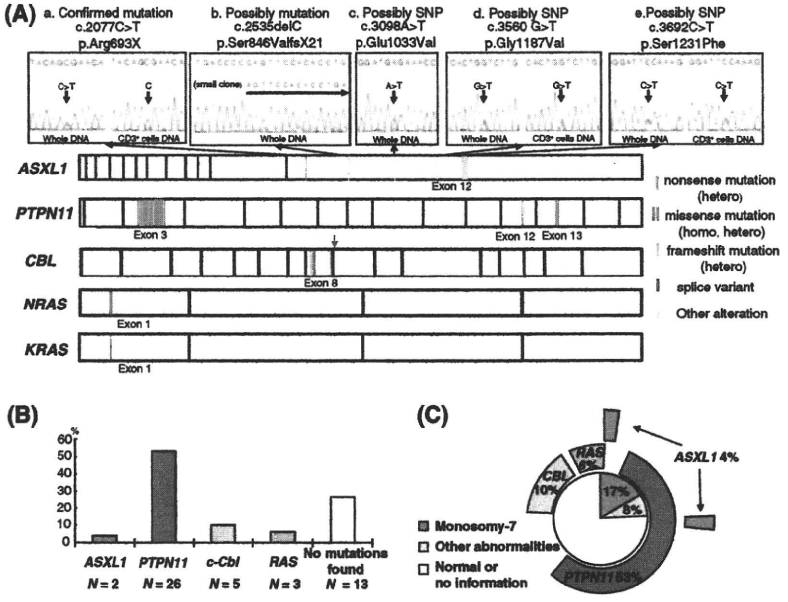

Fig 1. Mutational spectrum detected in JMML. High-density Affymetrix (250K) single nucleotide polymorphism array (SNP-A) karyotyping was performed on 49 patients with JMML and chromosomal lesions annotated. *PTPN11* (exon 2, 3, 4, 7, 8, 12 and 13), *NRAS*, *KRAS* (codons 12, 13 and 61), *TET2* (exon 3-11), *CBL* family members (exon 8 and 9 of *CBL*, exon 9 and 10 of *CBLB*, exon 7 and 8 of *CBLC*), *IDH1* (exon 4) and *ASXL1* (exon 12) were sequenced. For confirmation of somatic nature of observed mutations, immunoselected CD3+ cells were used as a germ line control. (A) Topography of mutations in *ASXL1*, *PTPN1*, *CBL*, *NRAS* and *KRAS* in JMML. Phenograms of nucleotide sequence alterations of *ASXL1* are shown. (B) Prevalence of each mutation in JMML. Percentage of each gene mutated cases in 49 JMML are shown. (C) Overlap between karyotype abnormalities and each mutation in JMML. Cytogenetic aberrations were identified by metaphase cytogenetics. Two cases with *ASXL1* mutations also harboured *PTPN11* mutation or monosomy-7, respectively.

frequent loss of heterozygosity (LOH) of 4q24 and an index microdeletion, mutations in *TET2* have been found in 40–50% of patients with CMML (Jankowska *et al*, 2009). Laboratory findings suggest that *TET2* mediates the hydroxylation of methylated cytosine residues in CpG islands and may thereby be a determinant of epigenetic instability (Tahiliani *et al*, 2009). However, unlike *CBL* mutations, *TET2* mutations have not been found in JMML and as a consequence, cases without a pathogenic mutation still constitute approximately 30% of patients with JMML (Muramatsu *et al*, 2009). While chromosomal abnormalities, particularly monosomy-7, are present in some of these cases, other pathogenetic mutations are likely and may involve genes within and outside of regions affected by LOH.

Recently, novel mutations involving isocitrate dehydrogenase 1 (*IDH1*) and the homologous gene *IDH2* have been identified in myeloproliferative neoplasms (MPN). *IDH1* and *IDH2* mutations are reported in 10–20% of de novo acute myeloid leukaemia (AML) (Mardis *et al*, 2009; Ward *et al*, 2010), and also in patients with secondary AML (sAML) that evolved from MPN (Green & Beer, 2010). These mutations are thought to lead to increased production of a pathological metabolite, 2-hydroxyglutarate (2HG), which contributes to malignant progression by yet an unknown mechanism.

In CMML, additional sex comb-like 1 (*ASXL1*) mutations have been found in approximately 40% of patients. This mutation has also been detected in a smaller proportion (10%) of patients with myelodysplastic syndrome (MDS), MPN and AML (primary AML 5%, secondary AML 50%) (Carbuccia *et al*, 2009; Gelsi-Boyer *et al*, 2009). *ASXL1* mutations are restricted to exon 12. ASXL1 cooperates with KDM1A (LSD1) in transcriptional repression, presumably by removing H3K4 methylation, an active histone mark, but not a repressive H3K9 methylation mark, recognized by HP1 (Lee *et al*, 2010). Based on evidence that some of the mutations are shared in related classes of myeloid malignancies, in particular CMML and JMML, we hypothesized that mutations in *IDH1*, *IDH2*, and *ASXL1* could also be present in children with JMML.

Results and discussion

We studied 49 children with JMML. Written informed consent for sample collection was obtained from the patients' parents according to established institutional protocols. Molecular analysis of the mutational status was approved by the Ethics Committee of Nagoya University Graduate School of Medicine. The diagnosis of JMML was based on the internationally accepted criteria (Niemeyer *et al*, 1998) and excluded patients with Noonan syndrome.

By conventional metaphase cytogenetics, chromosomal aberrations are found in only 25% of JMML patients. Using single nucleotide polymorphism arrays (SNP-A) as a

Table I. Clinical and molecular data in 49 JMML patients.

Case no.	Sex	Age (months)	Hb (g/l)	HbF (%)	WBC ($\times 10^9$/l)	Monocyte (%)	Platelets ($\times 10^9$/l)	Myeloblast (%)	SCT	Survival (month)	Karyotype	SNP-A	*ASXL1*	*PTPN11*	*CBL*	*RAS*
1	M	41	97	34·5	48·5	10	49·0	2	–	3, Dead	46,XY,inv(4)(p14p16)	–	WT	MT	WT	WT
2	M	65	49	32·4	15·3	17	26·0	17	–	4, Dead	46,XY	19p13.3 loss	WT	MT	WT	WT
3	F	66	78	3	32·2	46	58·0	2	+	6, Dead	45,XX,-7	monosomy-7, 12p13.2 loss	MT	WT	WT	WT
4	F	6	87	22·4	100	14	49·0	4	+	216, Alive	46,XX	–	WT	WT	WT	WT
5	M	58	89	1	11·8	14	13·0	25	–	10, Dead	45,XY,-7	monosomy-7, trisomy 21	WT	WT	WT	WT
6	F	47	78	17·7	85·5	37	21·0	7	+	10, Dead	46,XX	–	WT	WT	WT	WT
7	M	49	114	11·3	16·8	7	36·0	3	–	19, Dead	46,XY	–	WT	MT	WT	WT
8	M	27	94	46·1	72·9	10	25·0	4	+	8, Dead	48,XY,+X,+13 / 45,X,-Y	–	WT	MT	WT	WT
9	M	29	106	14·9	10·9	26	8·9	6	+	3, Dead	45,XY,-7	monosomy-7	WT	MT	WT	WT
10	M	61	120	55·4	12·2	9	8·0	1	+	116, Alive	46,XY	–	WT	MT	WT	WT
11	M	53	85	24·7	15·1	6	29·4	5	+	48, Alive	46,XY	–	WT	WT	WT	WT
12	M	36	109	43·9	19·4	16	42·0	3	+	84, Alive	46,XY	–	WT	WT	WT	WT
13	M	34	69	28·4	15·7	20	1·4	35	+	9, Dead	46,XY	–	WT	MT	WT	WT
14	M	56	120	9	58·5	26	102·0	2	+	6, Dead	45,XY,-7	monosomy-7, 1p31.3 gain	WT	MT	WT	WT
15	F	36	64	30·5	23·9	30	29·0	6	+	6, Dead	46,XX	17q11.2 loss, 6q21q25.3 loss	WT	WT	WT	WT
16	M	8	72	NA	36	18	85·0	2	+	10, Dead	46,XY	7p21.1 gain, 11q13.3q25 UPD	WT	WT	MT	WT
17	M	36	105	23·8	563	NA	59·0	NA	+	16, Dead	47,XY,+8	trisomy 8, 7q11.22 gain	WT	MT	WT	WT
18	M	24	84	35·8	12·6	18	38·0	3	+	63, Alive	46,XY	–	WT	MT	WT	WT
19	F	1	85	NA	55·4	25	49·0	10	–	11, Dead	46,XX	–	WT	WT	WT	WT
20	F	19	114	10·2	23·3	9	17·0	8	–	38, Alive	46,XX	5q23.1 loss	WT	WT	WT	WT
21	M	24	80	NA	49·8	6	8·0	19	+	19, Dead	45,XY,del(6)(q?),-20	–	WT	MT	WT	WT
22	M	35	109	49·3	14·7	7	123·0	NA	+	22, Alive	45,XY,-7	18q12.3 gain	WT	MT	WT	WT
23	M	12	89	2	50·1	9	320·0	3	–	21, Alive	46,XY	–	WT	WT	MT	WT
24	F	48	108	30·5	20·1	13	156·0	9	+	10, Dead	46,XX	–	WT	MT	WT	WT
25	M	11	86	1·1	16·8	22	65·0	0	–	84, Alive	46,XY	–	WT	WT	WT	MT
26	F	15	104	4·4	24·1	20	134·0	0	–	20, Alive	46,XX	–	WT	WT	WT	MT
27	F	15	69	NA	29·5	17	28·0	0	NA	NA	46, XX	11q12.1q25 UPD	WT	WT	MT	WT
28	F	16	111	20·8	56·2	8	175·0	1	+	22, Alive	46, XX	–	WT	MT	WT	WT
29	M	39	95	19	22·6	5	93·0	0	+	28, Alive	46, XY	10p11.23 gain	MT	MT	WT	WT
30	M	2	111	25·1	79·3	14	132·0	5	+	33, Alive	46, XY	–	WT	WT	WT	MT
31	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	–	WT	MT	WT	WT
32	M	20	116	56·1	33	16	53·0	1	+	24, Alive	46, XY	–	WT	MT	WT	WT
33	F	45	87	21·9	81·5	14	23·0	5	NA	NA	46, XX	–	WT	WT	WT	WT
34	F	18	NA	NA	NA	NA	NA	NA	+	35, Alive	46, XX	–	WT	WT	WT	WT
35	F	11	118	2·7	14·2	43	31·0	0	+	35, Alive	45, XX, -7	monosomy-7	WT	WT	WT	WT
36	M	28	NA	NA	NA	NA	NA	NA	+	36, Alive	45, XY, -7	monosomy-7, 8p21.2 loss	WT	MT	WT	WT
37	F	29	89	55	16	18	23·0	11	+	4, Dead	45, XX, -7	monosomy-7	WT	MT	WT	WT
38	F	13	92	3·5	20·8	14	65·0	0	+	21, Alive	46, XX	11q23.3q25 UPD	WT	WT	MT	WT
39	M	7	64	NA	NA	NA	NA	NA	NA	NA	NA	1q44 gain	WT	MT	WT	WT

Table I. (Continued).

Case no.	Sex	Age (months)	Hb (g/l)	HbF (%)	WBC ($\times 10^9$/l)	Monocyte (%)	Platelets ($\times 10^9$/l)	Myeloblast (%)	SCT	Survival (month)	Karyotype	SNP-A	*ASXL1*	*PTPN11*	*CBL*	*RAS*
40	M	18	83	15	51	6	48·0	1	+	18, Alive	46, XY	5q31.3 loss	WT	MT	WT	WT
41	M	2	76	37·4	59·5	17	59·0	2	–	1, Dead	46, XY	–	WT	MT	WT	WT
42	M	2	102	NA	10·5	18	71·0	6	+	5, Dead	46, XY	–	WT	MT	WT	WT
43	F	10	92	7·6	43·1	15	84·0	2	–	1, Dead	46, XX	11q23.3q25 UPD	WT	WT	MT	WT
44	M	6	137	NA	23·8	21	16·0	NA	–	7, Dead	46,XY	1q25.3 loss	WT	WT	WT	WT
45	M	41	99	32	49·9	8	19·0	1	+	14, Alive	46, XY	–	WT	MT	WT	WT
46	M	1	114	54	126·2	13	116·0	1	+	12, Alive	46, XY	2p22.1 loss	WT	WT	WT	WT
47	M	75	100	62	41	49	105·0	1	+	12, Alive	46, XY	15q26.3 gain	WT	MT	WT	WT
48	M	22	99	21	35·6	22	132·0	2	+	9, Alive	46, XY	17q11.2q25.3 UPD	WT	WT	WT	WT
49	M	65	113	28	31·9	9	75·0	10	–	1, Alive	46, XY	17q11.2 loss	WT	MT	WT	WT

NA, not available; SCT, stem cell transplantation; SNP-A, single nucleotide polymorphism arrays; WT, wild type; MT, mutant type.

karyotyping tool, we found chromosomal abnormalities in 49% of cases; UPD11q23, UPD17q or monosomy-7/7q- were present in 24% of cases (Muramatsu *et al*, 2009).

NF1 features, consistent with the presence of *NF1* mutations, were present in additional 2 patients. Recurrent del(7) in JMML could also be indicative of the presence of putative hemizygous mutations affecting specific genes on this chromosome. Mutations in genes involved in RAS pathway, including *KRAS*, *NRAS* and *PTPN11* were found in a significant proportion of JMML patients (59%). In contrast, these mutations were less frequently encountered in patients with the adult phenotypic counterpart of JMML, CMML. Based on the identification of *CBL* mutations in around 15% of CMML and other MDS/MPN cases (Makishima *et al*, 2009), we sequenced cases of JMML and have identified *CBL* mutations in 5/49 JMML cases. Both homozygous (1/49; due to UPD involving 11q23) and heterozygous (4/49) *CBL* mutations were found. However, unlike in CMML (Jankowska *et al*, 2009), *TET2* mutations were not detected in our JMML cohort, consistent with the total absence of LOH4q. Similarly, while advanced cases of CMML and sAML due to MPD may contain exon 4 *IDH1/2* mutations, they were totally absent in 49 JMML patients analysed in this study.

When exon 12 of *ASXL1* was sequenced in our cohort of JMML patients, we found 2 heterozygous mutations (4%): a nonsense (c.2077C>T p.Arg693X) and a frameshift (c.2535delC p.Ser846ValfsX21) mutation (Fig 1A). That these mutations prevent proper transcription into RNA suggests they represent somatic and not germline events. $CD3^+$ cells were used to confirm that the frameshift change was absent in the germline DNA; the non-sense mutation could not be confirmed. Neither mutation has been previously reported. LOH20q11 corresponding to *ASXL1* locus was not detected in any of the cases studied, indicating that the corresponding mutations were heterozygous. We have also identified a number of polymorphisms present in both tumour cells and $CD3^+$ cells (Fig 1A). The significance of these polymorphisms remains unclear but some have been observed in healthy controls.

Clinical analysis of patients with *ASXL1* mutations did not highlight any specific shared phenotypic features (Table I). Both cases presented with chromosomal abnormalities (none involving the *ASXL1* locus). In one case, monosomy-7 was detected in both metaphase cytogenetics and SNP-A. By SNP-A karyotyping, additional 12p13.2 loss was found in this case, while 10p11.23 gain was found in the second case. Patients affected by *ASXL1* mutations had a low myeloblast count in the marrow (2%, 0%) but only one displayed a significant monocytosis. Both patients underwent stem cell transplantation (SCT): patient 1 died 6 months after diagnosis, while the second patient is well 28 months after diagnosis.

ASXL1 mutations detected in JMML induce truncation of the protein downstream of the ASXH domain and a consequent loss of the PDH domain, suggested to be functionally important in the tumour suppressor function of *ASXL1*. While a loss of function mouse model of *ASXL1* showed that it is

required for normal haematopoiesis, a MDS or AML phenotype was not observed (Fishe *et al*, 2010). These finding may also suggest that *ASXL1* mutations may contribute to the pathogenesis of haematologic malignancies through gain-of-function and consequently murine knock-in models may be needed for investigation of their consequences. In contrast to *PTPN*, *NRAS*, *KRAS* and *CBL* mutations, which were mutually exclusive, one patient with an *ASXL1* mutation had also a *PTPN11* mutation. Consequently, it is possible that *ASXL1* mutations play a cooperative role, for example, in disease progression. Because ASXL1 function is related to the regulation of epigenetic inactivation patterns, theoretically, mutations of this gene may affect expression of genes involved in RAS signalling networks or *CBL*, which can increase activity of RAS signalling thought to be responsible for GM-CSF sensitivity of JMML progenitor cells. Of note is that this characteristic feature was present in all 49 JMML cases, including 30% of cases in which no mutations were found.

Conflict of interest

The authors declare no conflict of interest.

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

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Juvenile myelomonocytic leukemia (JMML) is a rare pediatric myeloid neoplasm characterized by excessive proliferation of myelomonocytic cells. When we investigated the presence of recurrent molecular lesions in a cohort of 49 children with JMML, neurofibromatosis phenotype (and thereby *NF1* mutation) was present in 2 patients (4%), whereas previously described *PTPN11*, *NRAS*, and *KRAS* mutations were found in 53%, 4%, and 2% of cases, respectively. Consequently, a significant proportion of JMML patients without identifiable pathogenesis prompted our search for other molecular defects. When we applied single nucleotide polymorphism arrays to JMML patients, somatic uniparental disomy 11q was detected in 4 of 49 patients; all of these cases harbored RING finger domain *c-Cbl* mutations. In total, *c-Cbl* mutations were detected in 5 (10%) of 49 patients. No mutations were identified in *Cbl-b* and *TET2*. *c-Cbl* and *RAS* pathway mutations were mutually exclusive. Comparison of clinical phenotypes showed earlier presentation and lower hemoglobin F levels in patients with *c-Cbl* mutations. Our results indicate that mutations in *c-Cbl* may represent key molecular lesions in JMML patients without *RAS*/*PTPN11* lesions, suggesting analogous pathogenesis to those observed in chronic myelomonocytic leukemia (CMML) patients. (Blood. 2010;115:1969-1975)

Introduction

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

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

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

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

Methods

Patients

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

Table 1. Characteristics of JMML patient cohort

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

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

SNP-A karyotyping analysis

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

Bioinformatic analysis

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

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

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

To screen for *PTPN11* mutations, we polymerase chain reaction amplified genomic DNA corresponding to exons 2, 3, 4, 7, 8, 12, and 13 as previously reported.[12,30,31] *NRAS* and *KRAS* mutations in codons 12, 13, and 61 were identified as previously described[32,33] and were confirmed by sequencing. To screen patients for mutations in E3 ubiquitin ligase genes and *TET2*, direct genomic sequencing of exons constituting the RFD of *Cbl* family members (exons 8 and 9 of *c-Cbl*, exons 9 and 10 of *Cbl-b*, exons 7 and 8 of *Cbl-c*, and exons 3-11 of *TET2*) was performed. For sequencing, 250 ng of polymerase chain reaction product, 3 μM original forward or reverse primer, 2 μL of Big Dye Version 3.1 (Applied Biosystems), and 14.5 μL of deionized H_2O were amplified under the following conditions: 95°C (2 minutes) followed by 25 cycles of 95°C (10 seconds), 50°C (5 seconds), and 60°C (4 minutes). Sequencing was performed as previously described.[22]

GM-CSF hypersensitivity assay

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

Statistical analysis

When appropriate, Kaplan-Meier statistics were applied to assess survival. For comparison of the frequency of mutation or other clinical features between disease groups, categoric variables were analyzed using the Fisher exact test and continuous variables were tested using the Mann-Whitney *U* test.

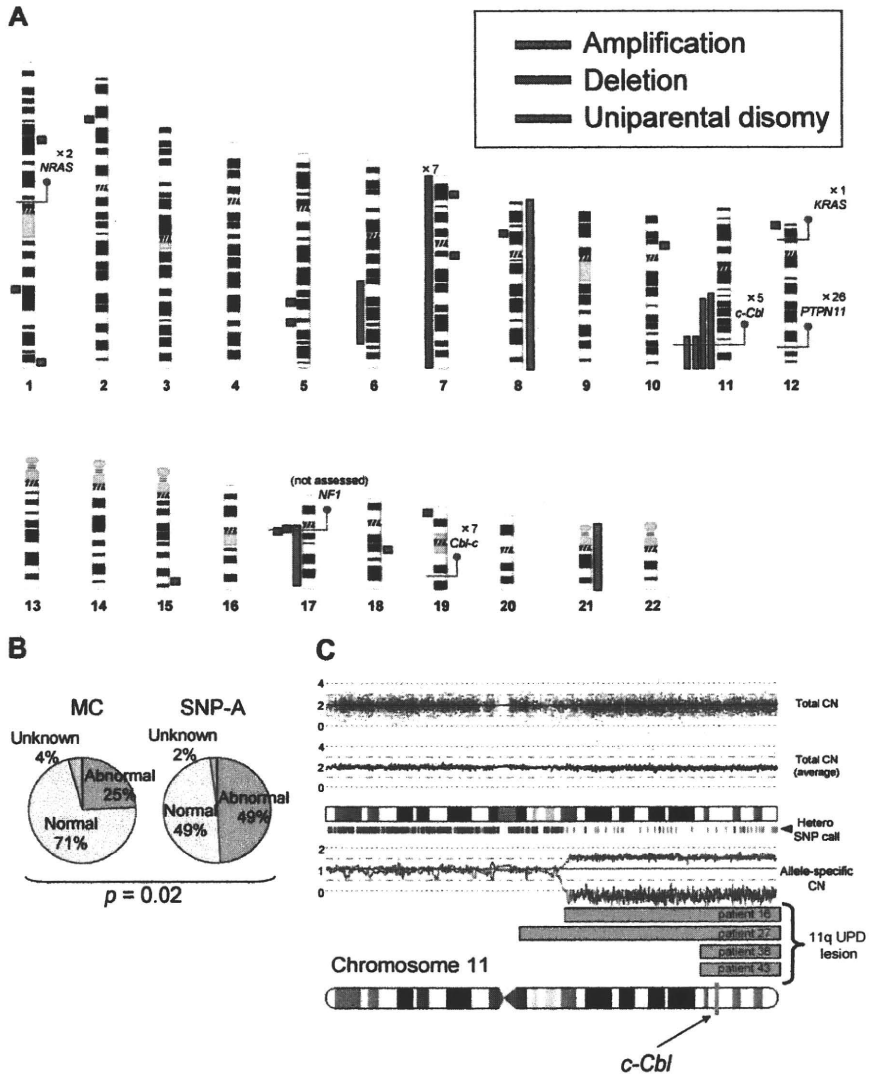


Figure 1. Single nucleotide polymorphism array–based karyotyping of JMML. (A) Genomic distribution and type of lesion identified in patients with JMML by SNP-A analysis. Green bar represent amplification, red shows deletion, and blue corresponds to UPD. Red lines pinpoint the locus of genes discussed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

Results

Cytogenetic and clinical characterization of JMML patients

First, we performed SNP-A– and metaphase cytogenetics–based analyses. Using conventional metaphase cytogenetics, chromosomal aberrations were found only in a minority of patients (25%). SNP-A–based karyotyping confirmed the results of metaphase cytogenetics, including the presence of monosomy 7 in 7 patients and trisomy 8 in 1 patient. However, due to increased precision and ability to detect copy-neutral loss of heterozygosity of SNP-A, additional lesions were identified by SNP-A in 24 (49%) of 48 patients, including trisomy 21 not detected by metaphase cytogenetics (MC) in 1 patient and microdeletions in 9 patients (Figure 1A), including 1q25.3 (patient 44), 2p22.1 (patient 46), 5q23.1 (patient 20), 5q31.3 (patient 40), 6q21q25.3 (patient 15), 8p21.2 (patient 36), 12p13.2 (patient 3), 17q11.2 (patients 15, 49), and 19p13.3 (patient 2). We also detected microamplifications in 7 patients (Figure 1A), located at 1p31.1 (patient 14), 1q44 (patient 39), 7p21.1 (patient 16), 7q11.22 (patient 17), 10p11.23 (patient 29), 15q26.3 (patient 47), and 18q12.3 (patient 22). The shared copy number–altering lesions included monosomy 7 and loss of 17q11.2, which contained the *NF1* locus. Although we were unable to confirm the somatic nature of the submicroscopic defects due to lack of germline DNA, these lesions did not overlap with copy number variations present in internal control cohort and publicly available databases. Most significantly, we identified UPD in 5 patients (Figure 1A). UPD11q was found in 4 patients, all regions overlapping from 11q23.3 to the telomere. This commonly affected region contained the *c-Cbl* locus (Figure 1A,C). The region of UPD at 17q contained the *NF1* locus and corresponded with clinical neurofibromatosis features. Overall, compared with the results of MC, SNP-A identified significantly more genetic abnormalities (25% vs 49%; $P = .02$; Figure 1B).

Table 2. Patients' mutational status

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

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

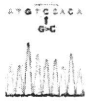
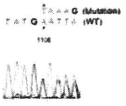
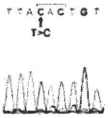
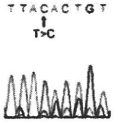
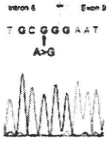
Mutational analysis of patients with JMML

After defining chromosomal defects associated with JMML, we performed mutational analysis of the genes known to be affected by mutations in JMML. *PTPN11* mutations were found in 26 (53%) of 49, whereas *NRAS* and *KRAS* mutations were found in 2 (4%) of 49 and 1 (2%) of 49, respectively (Table 2). None of the patients screened show the presence of *TET2* mutations, previously shown to be present in a significant proportion of patients with MDS/MPD, including CMML.[21] Excluding patients with a neurofibromatosis phenotype, 18 (37%) of 49 of patients did not show any of the known pathogenic defects occurring in JMML.

Identification of *Cbl* gene family mutations in JMML

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

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

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

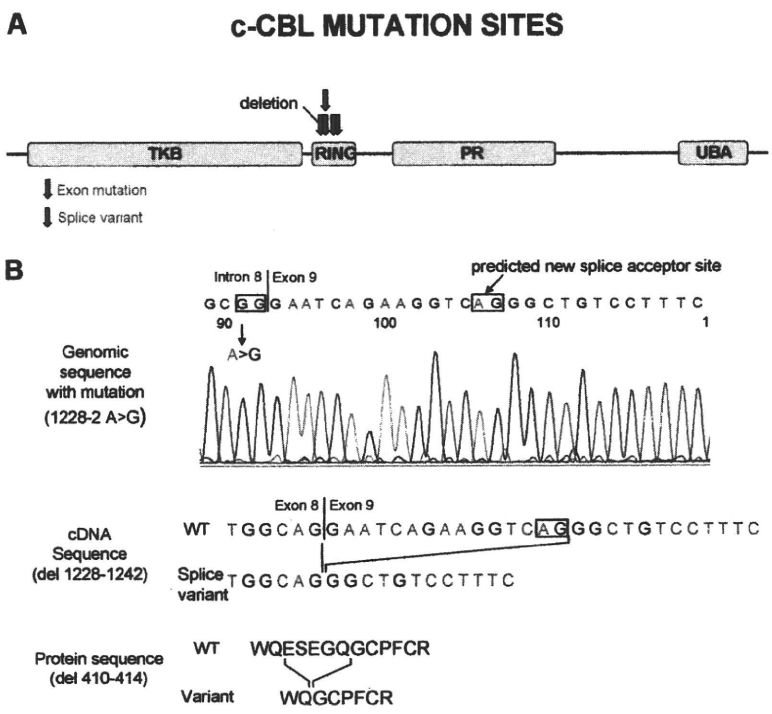


Figure 2. Site of the *c-Cbl* mutations and predicted product of splice variant in the intron 8 splice acceptor site. (A) Localization of the c-Cbl mutations within the predicted protein product. Red arrows show the site of mutations in exon, and blue arrows show the site of splice variant. (B) In patient 48, a homozygous mutation was seen in the intron 8 splice acceptor site of *c-Cbl*. According to http://genome.cbs.dtu.dk/services/NetGene2,[34] this mutation may result in a splice variant, leading to a shorter transcript in RF domain.

When we investigated our cohort for the presence of *Cbl-c* mutations, we found heterozygous frameshift nucleotide variation (1256 insertion C; patients 7, 12, 14, 29, 33, 38, and 46; data not shown). However, *Cbl-c* mutational status of germline though sequencing of nonclonal $CD3^+$ lymphocytes in those patients showed the same frameshift mutation. Consequently, these *Cbl-c* nucleotide exchanges represent rare polymorphisms.

GM-CSF hypersensitivity assay

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

Clinical features associated with *Cbl* gene family mutations

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

We did not find any distinctive morphologic features of patients with *Cbl* gene family mutations and no differences were present in the blood counts at initial presentation. Other variables studied (sex, the presence of cytogenetic abnormalities) also did not differ between patients grouped according to mutational status. However, patients with mutant *c-Cbl* compared with those with wild-type constellation showed earlier presentation (median age at diagnosis, 12 months vs 29 months, $P = .037$) and lower median hemoglobin F (HbF) percentage (3.5% vs 24.9%, $P = .02$), previously shown to correlate with less favorable prognosis.[1,33,35-40] Low HbF values in *c-Cbl* mutant cases were not attributable to monosomy 7, absent in this patient cohort. The probability of 2-year overall survival of *c-Cbl* mutant patients (50.0%; 95% confidence interval [CI], 25.0%-75.0%; n = 4) was similar to that of patients without *c-Cbl* mutations (50.4% [95% CI, 42%-59%]; n = 41). Similarly, when

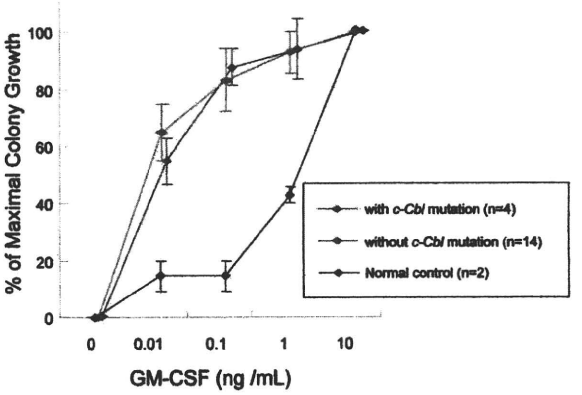


Figure 3. GM-CSF hypersensitivity assay. Colony counts are expressed as percentage of maximal numbers of CFU-GM (colony counts cultured with each concentration of GM-CSF/colony counts cultured with 10 ng/mL GM-CSF). The similar GM-CSF hypersensitivity was seen in JMML patients with or without *c-Cbl*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

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

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

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

HbF indicates hemoglobin F.

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

Discussion

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

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

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

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

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

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

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

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

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Authorship

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

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