GENETIC TYPING OF CBL, ASXL1, RUNX1, TET2, AND JAK2 IN JUVENILE MYELOMONOCYTIC LEUKEMIA (JMML) REVEALS A GENETIC PROFILE DISTINCT FROM CHRONIC MYELOMONOCYTIC LEUKEMIA (CMML).

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Keywords: JMML, CMML, ASXL1, CBL, TET2, RUNX1, JAK2

Running title: JMML and CMML have distinct genetic profile
ABSTRACT

JMML and CMML are rare myelodysplastic/myeloproliferative neoplasms occurring at both ends of life. To investigate relationships between JMML and CMML, genes recently involved in CMML were studied in 68 JMML patients. Mutations in TET2, RUNX1, and JAK2V617F are involved in myelodysplastic and/or myeloproliferative syndromes, and more specifically in CMML but were not found in JMML. Pangenomic analysis by SNP-array showed no abnormality at these loci. Three frameshift mutations of ASXL1 leading to a truncated protein were found in 3 patients (4%) with late onset JMML displaying also RAS activating mutations. Homozygous mutations of CBL with 11q loss of heterozygosity were found in 5 (7%) JMML. CBL substitutions were different from those reported in CMML, exclusive from other RAS activating mutations, and were germline in all patients. Overall, the pattern of genetic lesions observed in JMML differed from that of CMML. Although signalling deregulation is involved in CMML, transcriptional deregulation seems to play a pivotal role, with mutation of RUNX1, ASXL1 or TET2. Conversely, none of these genes involved in transcription or chromatin remodelling was found to be significantly altered in JMML, while CBL mutations confirm the central role of RAS and growth factor signalling deregulation in JMML.
INTRODUCTION

Juvenile myelomonocytic leukaemia (JMML) is a rare and aggressive haematological malignancy classified by the WHO (World Health Organization) into combined myelodysplastic and myeloproliferative neoplasms (MDS/MPN). JMML affects infants and young children and represents about 2-3% of children leukaemia (Flotho et al, 2007; Koike and Matsuda, 2008). It is characterised by an excess of monocyte/macrophage proliferation with subsequent infiltration of haematopoietic tissues leading to bone marrow failure. A particularity of JMML is its frequent association with an underlying predisposing genetic syndrome, neurofibromatosis type 1 or Noonan syndrome (NS). JMML responds poorly to chemotherapy and the only curative treatment is allogenic bone marrow transplantation, with a very high relapse rate (35-40%). Up to 30% of patients progress to acute myeloid leukaemia (AML) (Castro-Malaspina et al, 1984; Lauchle et al, 2006). Most patients die from respiratory and organ failure. However, clinical course is surprisingly heterogeneous and spontaneous recovery has even been reported in some patients (Matsuda et al, 2007). The excess of monocytes observed in patients with JMML is due to hypersensitivity of myeloid progenitors to granulocyte-macrophage colony-stimulating factor (GM-CSF). This hypersensitivity is mediated by the RAS signalling pathway which is pathologically activated in JMML due to point mutations in several genes: PTPN11 (Tartaglia et al, 2003; Yoshida et al, 2009), NRAS, KRAS (Flotho et al, 1999; Niemeyer et al, 1997), NF1 (Shannon et al, 1992; Shannon et al, 1994) or CBL (Loh et al, 2009).

Chronic myelomonocytic leukaemia (CMML) is also a rare and severe MDS/MPN but it is observed in the elderly. CMML share with JMML several diagnostic criteria such as splenomegaly and persistent blood monocytes and is also characterised by a wide heterogeneity of presentation and clinical course (Emanuel, 2008). As in JMML, RAS gene mutations are observed in almost one third of CMML (Onida, et al, 2002, Sanada, et al 2009) but the pathogenesis of CMML is less understood. However, in the past two years, a number of new genes have been reported to play a major role in CMML. These genes include TET2 (Ten-eleven translocation-2), RUNX1 (Runt-related transcription factor 1 isoform, also known as AML1), JAK2 (Janus kinase 2), ASXL1 (additional sex combs like 1), and CBL (c-CBL; Casitas B-lymphoma).

TET2 encodes a methylcytosine dioxygenase that may contribute to epigenetic regulations via the generation of hydroxymethylcytosines. TET2 is widely expressed in myeloid cells. Mutations scattered throughout the coding sequence were recently identified in different myeloid malignancies and in 42% of CMML (Abdel-Wahab et al, 2009; Delhommeau et al,
2009; Jankowska et al, 2009; Kosmider et al, 2009; Tefferi et al, 2009). RUNX1 encodes a transcription factor, which is essential for normal haematopoiesis and differentiation. Mutations in RUNX1 have been found in 23% of CMML RUNX1 (Ernst et al, 2010; Gelsi-Boyer et al, 2008; Kuo et al, 2009). They usually target the Runt domain. The JAK2V617F mutation is found in 7% of CMML (Jelinek et al, 2005; Renneville et al, 2006; Steensma et al, 2005; Szpurka et al, 2006; Tyner et al, 2009). No other JAK2 mutation has been reported in this disease. ASXL1 belongs to the polycomb gene family containing three identified members (ASXL1, 2, 3) that encode proteins regulating chromatin remodelling. Mutation of the ASXL1 gene has been reported in 38% of CMML (Boultwood et al, 2010a; Gelsi-Boyer et al, 2009). Previously reported mutations of ASXL1 in haematological malignancies were found exclusively within exon 12 (Tefferi, 2010), which encodes more than half of the coding sequence and all C-terminus interacting motifs. CBL protein is a member of the CBL family of E3 ubiquitin ligases also displaying molecular adaptor functions (Kales et al, 2010). CBL is involved in the down regulation of transduction pathways induced by the tyrosine kinase receptors of several growth factors. CBL contains a tyrosine kinase binding (TKB) domain and a Zinc-binding RING finger domain separated by a short linker sequence. CBL mutations have been reported in various myeloid neoplasms including 12% of CMML (Dunbar et al, 2008; Grand et al, 2009; Loh et al, 2009; Makishima et al, 2009; Sanada et al, 2009). They all target the RING and linker domains, coded by exons 7 to 9, and abrogate the ubiquitin ligase activity of CBL.

In order to have a better insight into the relationship between JMML and CMML, and possibly discover new genes involved in JMML pathogenesis, we screened mutations of these genes in a cohort of 68 patients with JMML. Mutation frequencies found in our patients with JMML were then compared to those reported in the literature for CMML.
PATIENTS AND METHODS

Patients and samples

Sixty-eight unselected patients with JMML referred to our lab between 1991 and 2010 were included in the study. All patients fulfilled the JMML criteria reported recently by Chan et al. (Chan, et al. 2009). The median age at diagnosis was 13 months. Informed consent of the parents according to the Helsinki agreement was obtained. Bone marrow aspirates (n=56) or peripheral blood (n=12) were collected on EDTA at diagnosis. Progression to AML during the course of the disease occurred in 20/68 (29%) patients. DNA was available at the time of AML in 6 of these patients.

When possible, fibroblasts were also collected. Karyotype was systematically performed using standard procedures. Genomic DNA was extracted from leucocytes preparations using Qiagen Mini Kit (Qiagen Gmbh, Hilden, Germany).

Mutational analysis

Mutation screening was performed for genes known to be associated with JMML (NRAS, KRAS, PTPN11, NF1) (Pérez et al, 2010) and for genes recently shown to be involved in CMML (CBL exons 7, 8, 9, ASXL1 exon 12, RUNX1 exons 3 to 8, TET2 exons 3 to 11). RUNX1 and TET2 mutation screening were performed in 65/68 patients only. Genetic typing of JMML was performed on genomic DNA by bi-directional sequencing of exons and their flanking intron-exon boundaries. Direct sequencing of PCR products was performed using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI, Foster City, CA, USA). Reaction products were run on an automated capillary sequencer (ABI 3130 Genetic Analyser, ABI). Sequences were aligned using Seqscape® analysis software (ABI) and compared with the reference sequences for genomic DNA and mRNA. GenBank accession number for genomic reference sequences are as follows: ASXL1 NM_015338, RUNX1 (AML1) NM_001754, CBL NM_005188, TET2 NM_017628. ASXL1 insertion/deletions mutations within the G stretch were checked by fragment analysis. The mutated sequence was amplified by PCR using a 6-FAM labelled forward primer (5’-GGACCCTCGCAGACATTAAA-3’) and a reverse primer (5’-CACCACCATCACCACTGCT-3’) flanking the G stretch. PCR were conducted using a proofreading Taq polymerase with 3’-5’ exonuclease activity (expand long template PCR system, Roche diagnostics, Meylan, France). PCR products were run on an automated capillary sequencer (ABI 3130 Genetic Analyser, ABI) and sized using the Genescan® analysis software (ABI). JAK2V617F mutation
was detected by a SNP genotyping assay using real-time PCR and Taqman technology as described (Kiladjian et al, 2006).

Previous implication of the mutations in cancer was checked by consulting the Catalogue for Somatic Mutations in Cancer (http://www.sanger.ac.uk/genetics/CGP/cosmic). Presence of Single nucleotide polymorphism (SNP) was checked by consulting the Ensembl genome browser (http://www.ensembl.org). Prediction of functional effects of amino acid substitutions on the function and structure of proteins were achieved by interspecies alignments and using dedicated prediction softwares (Table I).

Mutation frequencies found in our patients with JMML were then compared to those reported in the literature for CMML. Cumulative frequencies of mutations were calculated in CMML from studies reporting mutation data for RAS (Gelsi-Boyer et al, 2008; Hirsch-Ginsberg et al, 1990; Onida et al, 2002; Padua et al, 1998; Sanada et al, 2009; Tyner et al, 2009), TET2 (Abdel-Wahab et al, 2009; Delhommeau et al, 2009; Jankowska et al, 2009; Kosmider et al 2009, Tefferi, et al 2009), RUNX1 (Ernst et al, 2010; Gelsi-Boyer et al, 2008; Kuo et al, 2009), JAK2 (Jelinek et al, 2005; Renneville et al, 2006; Steensma et al, 2005; Szpurka et al, 2006; Tyner et al, 2009), ASXL1 (Boultwood et al, 2010a; Gelsi-Boyer et al, 2009) and/or CBL (Dunbar et al, 2008; Grand et al, 2009; Loh et al, 2009; Makishima et al, 2009; Sanada et al, 2009). Only studies reporting data for 5 CMML or more were taken into account.

**SNP-array analysis**

SNP-array analysis was performed for 51/68 patients. Tumor (Bone marrow cells or peripheral blood mononuclear cells) and, when available, germline (fibroblast) DNA were hybridized to Affymetrix Genome-Wide Human SNP 6.0 Arrays (Affymetrix, Santa Clara, CA, USA) according to the manufacturer’s protocol. CEL files were created using the Genechip Command Console operating software and Genotyping console 2.1. The Partek Genomics Suite was used to detect possible loss of heterozygosity (LOH) and/or copy number changes (CNC) of the gene that were screened for mutations. Regions of CNC were detected using a Hidden Markov Model. LOH was assessed by comparing paired germinal and tumor samples using Partek LOH workflow.
RESULTS

Mutation screening of the genes classically associated with RAS activation in JMML was performed in the JMML cells of the 68 patients. RAS mutations were found in 23/68 (34%) patients, with NRAS mutations in 11 cases (17%) and KRAS mutations in 12 cases (18%). Mutations of PTPN11 were found in 30/68 cases (44%). Mutations of NF1 were present in all (3/3) cases with clinical manifestations of neurofibromatosis type 1 (4% of the total) and were always associated with LOH of the NF1 locus in the malignant sample. LOH of the NF1 locus was not observed in other patients. Altogether, a RAS activating mutation was found in 56/68 (82%) patients. These frequencies are in accordance with previously reported data (de Vries, et al 2010).

Sequencing of the TET2 complete coding sequence in 65 JMML showed no mutation. No copy number change or LOH was found at the TET2 locus (4q24) either.

Two RUNX1 heterozygous missense variants (p.T219M, p.G259K) that had not been previously reported were detected in two patients (Table I). The p.T219M substitution was found in a patient with neurofibromatosis type 1 and was inherited from his father. The p.G259K was also germline, as shown by its presence in the fibroblasts of the patient. Although these variants were not found in a series of 350 controls (Preudhomme et al, unpublished data), prediction softwares indicated a probable benign functional effect (Table I). Neither patients displaying these variants nor others showed RUNX1 LOH at 21q22.12 or copy number change. No clinical argument for familial RUNX1 haploinsufficiency was present in individuals with these variants. We therefore concluded that they are probable unreported rare polymorphisms without pathological significance.

All JMML patients were negative for JAK2V617F and no copy number change or LOH was observed at the JAK2 locus (9p24.1).

Three ASXL1 heterozygous nonsense mutations, c.1934delG (p.G645VfsX58), c.1934dupG (p.G646WfsX12), and c.2324T>G (p.L775X) were identified in 3 patients out of 68 (4%) (Fig 1). The presence of a delG or dupG mutations was confirmed by a PCR assay using a proofreading Taq polymerase. Fragment length analysis ruled out the possibility of artefacts due to a mismatch of the polymerase favoured by the G stretch (data not shown). The three frameshift mutations lead to a stop codon, with truncation of the C-terminus of the protein including the PHD finger, and the nuclear receptor box, which is predicted to interact with the retinoic acid receptor (Gelsi-Boyer et al, 2009). The main characteristics of these 3 patients are described in Table II. The three patients also had a RAS activating mutation (NRASG13D...
and PTPN11K72V and KRASQ61P respectively). Two had abnormal karyotypes, with monosomy 7 in one case and del12p13 in the other. Another common feature of these patients was a relatively high age at diagnosis of JMML (4 yrs 9 mo, 7 yrs 6 mo, 2 yrs 1 mo (for a median age at diagnosis of 13 mo for the whole cohort of patients). Although one patient suffered blastic transformation to AML with myelomonocytic differentiation, patients are still alive 6, nearly 3 years, and 6 mo after diagnosis. ASXL1 missense variants (p.E1102D, p.L1395V) were found in 2 other patients with JMML. p.E1102D has been reported once as non pathological SNP (Carbuccia et al, 2010) and once as a mutation (Szpurka et al, 2010), and p.L1395V has been reported as a mutation (Szpurka et al, 2010). Prediction software analyses were consistently in favour of non pathological SNP for both variants. This is in line with the presence of these variants in non tumoral cells (fibroblasts) of our 2 patients (Table I). ASXL1 mutations were screened at the time of blastic crisis in 6 patients that were negative for ASXL1 mutation at JMML presentation. No mutation was found in these samples.

Five homozygous mutations of CBL were found in leukaemia cells of 5/68 (7%) JMML patients (Table II). One patient had a c.1254C>G (p.F418L) mutation, one had a mutation affecting a splice site in intron 8, (c.1228-2A>G) and 3 had a c.1111T>C (p.Y371H) mutation. In all our JMML patients, copy neutral LOH due to acquired uniparental isodisomy (aUPD) of the 11q chromosomal region, encompassing the CBL locus (11q23.3) was demonstrated in leukaemia cells. CBL mutations were mutually exclusive from other RAS activating mutations. Sequencing of fibroblasts DNA showed the presence of a germline heterozygous mutation in all patients. All patients displaying a CBL mutation are alive (Table II).

**DISCUSSION**

In the past few years, mutations of CBL (Dunbar et al, 2008; Grand et al; 2009; Loh et al, 2009; Makishima et al, 2009, Sanada et al, 2009), RUNX1 (Ernst et al, 2010; Gelsi-Boyer et al, 2008; Kuo et al, 2009), TET2 (Abdel-Wahab et al, 2009; Delhommeau et al 2009, Jankowska, et al 2009, Kosmider, et al 2009, Tefferi, et al 2009), and ASXL1 (Boultonwood, et al 2010a, Gelsi-Boyer et al, 2009) genes have been identified as major genetic abnormalities in various myeloproliferative and/or myelodysplastic syndromes, and more specifically in CMML. JAK2V617F mutations have been also reported in CMML, although at a lower
frequency (Jelinek et al, 2005; Renneville et al, 2006; Steensma et al, 2005; Szpurka et al, 2006; Tyner et al, 2009).

Unlike what has been described for CMML, TET2 showed no mutation, and no copy number variation or LOH in our JMML, in line with recent reports (Jankowska et al, 2009; Muramatsu et al, 2009). RUNX1 mutations were not found either in our JMML patients. One case of JAK2V617F has been previously reported in JMML (Tono et al, 2005). The absence of mutation in our patients confirms that is a very uncommon event in JMML (Zecca et al, 2007).

Mutation of the ASXL1 gene has been reported in 38% of CMML (Boultwood et al, 2010a; Gelsi-Boyer et al, 2009) but also in 11% of myelodysplastic syndromes (MDS) (Boultwood et al, 2010a; Gelsi-Boyer et al, 2009), in 8% of myeloproliferative neoplasms (Carbuccia et al, 2009), in 17 to 25% of patients with AML (Boultwood et al, 2010a; Carbuccia et al, 2010), in 15% of chronic myeloid leukaemia (CML) (Boultwood et al, 2010b). A 20q deletion, encompassing the ASXL1 locus, has been reported to be associated in 2 cases with ASXL1 mutation (Boultwood et al, 2010a). These data suggest that ASXL1 represents a new tumor suppressor gene, the inactivation of which may have an important role in the molecular pathogenesis of malignant myeloid disorders. ASXL1 mutations lead to a truncated protein lacking the nuclear receptor box, which is predicted to interact with the retinoic acid receptor, and a PHD finger located at the extreme C terminus. Function of the human ASXL1 protein is poorly understood, but there is some evidence that ASXL1 may represent a component of DNA- and/or histone-modifying complexes. It is required for normal haematopoiesis and maintains both activation and silencing of Hox genes in flies and mice (Fisher et al, 2010). Noteworthy, the ASXL1 mutations identified in this study are both heterozygous frameshift mutations caused by deletion or duplication of a nucleotide located on a polyG stretch. This polyG stretch corresponds to a mutation hotspot in several malignant neoplasms, with the ASXL1 c.1934dupG (p.G646WfsX12) accounting for 53% of mutations in CMML, MDS, MPD, AML and CML. Instability of simple repetitive DNA sequences is involved in several cancer types, and is favoured by defects in the DNA mismatch repair (MMR) system. Therefore, the type of mutation observed in ASXL1 suggests a possible role for genetic instability. Increased genetic instability is a feature of tumor evolution. Interestingly, ASXL1 mutations have also been shown recently to be the commonest known mutations in advanced MDS, confirming a possible association with disease progression (Boultwood et al, 2010a). Although not formally demonstrated, it is generally assumed that RAS activating mutations are the initiating event in JMML (Flotho et al, 2007). In this respect, ASXL1 mutations, which were associated with RAS activating mutations, are possibly secondary mutations associated with JMML progression rather than initiation. This is in line with the fact that our 3 patients displayed additional genetic and/or cytogenetic alterations. Advanced
aged of our patients with ASXL1 mutation may also be consistent with a diagnosis at a relatively advanced stage of the disease. However, the presence of an ASXL1 mutation was not associated with a pejorative outcome in our patients (Table I). Moreover, ASXL1 mutations are not likely to play a major role in progression to AML since only one patient with ASXL1 mutation progressed to AML and no ASXL1 mutation was found in samples collected at the time blast crisis. During this work, two other heterozygous mutations of ASXL1 (p.Arg693X and p.Ser846ValfsX21) have been reported in JMML (Sugimoto et al, 2010) confirming the presence of ASXL1 mutations in about 3% of JMML. Similarly to our patients, those reported by Sugimoto et al. displayed cytogenetic abnormalities and advanced age at JMML onset. Interestingly, monosomy 7 and deletion at 12p13 were found associated to ASXL1 mutations in both studies, suggesting a possible cooperation between ASXL1 mutations and these abnormalities.

Mutations of CBL were found in 8% of our patients and were mutually exclusive of other RAS activating mutations, raising the proportion of mutated JMML to 90%. Our findings confirm the recurrence of the CBL Y371H mutation in JMML, in accordance with previous reports (Loh et al, 2009; Muramatsu et al, 2009; Shiba et al, 2010). Including ours, 40 CBL mutations have been reported so far in JMML, 19 (48%) target Y371 and 16 (40%) were p.Y371H substitutions. Mutations p.Y371C and p.Y371S have been described in one case of CMML each but the Y371H was never reported in CMML. Only one Y371H mutation has been reported so far in another myeloid malignancy (one patient with atypical CML was a compound heterozygous p.Y371H/p.R462X) (Grand et al, 2009). Thus, although both JMML and CMML display mutations in CBL, substitutions found in JMML are highly specific. The loss of heterozygosity associated with CBL mutations is in line with a tumor suppressor function of CBL. However, the strong association of a specific substitution with JMML suggests that loss of function may not be the only consequence of CBL mutations, in line with a recent study demonstrating a gain-of-function of CBL mutants in a CBL-/- background (Sanada, et al 2009). Interestingly, our 5 patients with CBL homozygous mutations in JMML showed germline heterozygous mutation of CBL. This probably explains the clinical presentation of these patients who displayed a variable combination of dysmorphic features, hyperpigmented skin lesions, microcephaly, and developmental delay. A detailed clinical description of three of these patients has been recently reported (Pérez et al, 2010). Thus germline CBL mutations (or “CBL syndrome”) represent a new inherited condition with predisposition to JMML, in addition to Noonan syndrome and neurofibromatosis type 1.

In conclusion, our study shows that genes that have been recently involved in CMML pathogenesis are not frequently mutated in JMML (Fig 2). CBL mutations are found at similar frequencies in both diseases. However, JMML-associated mutations are distinct from those
reported in CMML and are found in a context of syndromic JMML with germline heterozygous CBL mutation. Overall, the pattern of genetic lesions observed in JMML clearly differs from that of CMML (Fig 2). Although signalling deregulation is involved in CMML, as shown by the presence of CBL, RAS or JAK2 mutations, transcriptional deregulation seems to play a pivotal role with the frequent mutation of RUNX1, ASXL1 or TET2. Conversely, none of the genes involved in transcription and/or chromatin remodelling was found to be significantly altered in JMML, while the finding of CBL mutations confirms the central role of RAS and growth factor signalling deregulation in this disease.

Conflict of interest
The authors declare no conflict of interest

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FIGURE LEGENDS

**Figure 1**: Sequence electrophoregrams documenting ASXL1 mutations.

A. Patient 11: c.1934delG (p.G645VfsX58)

B. Patient 42: c.1934dupG (p.G646WfsX12)

C. Patient 67: c.2324T>G (p.L775X). Heterozygous point mutation is indicated by an asterix.

**Figure 2**: Mutation frequencies in JMML and CMML

The frequency of mutations found in our cohort of patients with JMML (black boxes) is compared with the cumulative frequencies reported in the literature for CMML (grey boxes). The percentage of cumulated number of patients analyzed is indicated for CMML. Cumulative frequencies of mutations were calculated from studies reporting at least 5 cases of CMML tested for RAS (Gelsi-Boyer et al, 2008; Hirsch-Ginsberg et al, 1990; Onida et al, 2002; Padua et al, 1998; Sanada et al, 2009; Tyner et al, 2009), JAK2 (Jelinek et al, 2005; Renneville et al, 2006; Steensma et al, 2005; Szpurka et al, 2006; Tyner et al, 2009), CBL (Dunbar et al, 2008; Grand et al, 2009; Loh et al, 2009; Makishima et al, 2009; Sanada et al, 2009), RUNX1 (Ernst et al, 2010; Gelsi-Boyer et al, 2008; Kuo et al, 2009), TET2 (Abdel-Wahab et al, 2009; Delhommeau et al, 2009; Jankowska et al, 2009; Kosmider et al, 2009; Tefferi et al, 2009) and/or ASXL1 (Boulwood et al, 2010a; Gelsi-Boyer et al, 2009).
### Table I. Description and predicting effect of novel SNP identified in ASXL1 and RUNX1

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<th>Genes</th>
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<th>Amino-acid</th>
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<th>Negative controls</th>
<th>Interspecies conservation</th>
<th>Grantham score&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Panter&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>ASXL1</td>
<td>12</td>
<td>29</td>
<td>c.3306G&gt;T</td>
<td>p.E1102D&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>45</td>
<td>Possibly deleterious</td>
<td>neutral</td>
<td>benign</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td></td>
<td>c.4183C&gt;G</td>
<td>p.L1395V&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1</td>
<td>yes</td>
<td>yes</td>
<td></td>
<td>32</td>
<td>neutral</td>
<td>neutral</td>
<td>benign</td>
</tr>
<tr>
<td>RUNX1</td>
<td>6</td>
<td>28</td>
<td>c.656C&gt;T</td>
<td>p.T219M</td>
<td>1</td>
<td>yes</td>
<td>yes</td>
<td></td>
<td>81</td>
<td>NA</td>
<td>pathological</td>
<td>benign</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>29</td>
<td>c.775C&gt;A</td>
<td>p.Q259K</td>
<td>1</td>
<td>yes</td>
<td>yes</td>
<td></td>
<td>53</td>
<td>neutral</td>
<td>neutral</td>
<td>possibly damaging</td>
</tr>
</tbody>
</table>

N: nucleotide; AA: amino-acid; NA: not available

<sup>a</sup> [http://www.genome.jp/dget-bin/www_bget?aa:x2:GRAR740104](http://www.genome.jp/dget-bin/www_bget?aa:x2:GRAR740104); the Grantham score is considered pathological if >100;
<sup>b</sup> [http://www.pantherdb.org/tools/csnpScoreForm.jsp](http://www.pantherdb.org/tools/csnpScoreForm.jsp)
<sup>d</sup> [http://genetics.bwh.harvard.edu/pph/](http://genetics.bwh.harvard.edu/pph/)
<sup>e</sup> reported as a non pathological SNP by Carbuccia et al (Carbuccia et al, 2010), and as a mutation by Szpurka et al (Szpurka et al, 2010)
<sup>f</sup> reported as a mutation by Szpurka et al (Szpurka et al, 2010)
<sup>g</sup> (Boultwood et al, 2010a)
<sup>h</sup> Preudhomme et al. (unpublished data)
Table II. Characteristics of patients with JMML showing *ASXL1* or *CBL* mutations

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age at diagnosis (in months)</th>
<th>RAS pathway mutation</th>
<th>Gene</th>
<th>Nucleotide</th>
<th>Amino acid</th>
<th>Karyotype at diagnosis</th>
<th>Hb g/dl</th>
<th>PI 10^9/L</th>
<th>WBC 10^9/L</th>
<th>% BM Blasts cells</th>
<th>HbF</th>
<th>BMT</th>
<th>Leukemic Transformation</th>
<th>Follow up (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
<td>M</td>
<td>57</td>
<td>PTPN11&lt;sup&gt;A72V&lt;/sup&gt;</td>
<td><em>ASXL1</em></td>
<td>c.1934dupG</td>
<td>p.G646WfsX12</td>
<td>45,XY,7 [16]</td>
<td>7</td>
<td>102</td>
<td>17</td>
<td>19</td>
<td>6.8% Elevated for age</td>
<td>yes</td>
<td>AML FAB4</td>
<td>Alive</td>
</tr>
<tr>
<td>67</td>
<td>F</td>
<td>25</td>
<td>KRAS&lt;sup&gt;G99G&lt;/sup&gt;</td>
<td><em>ASXL1</em></td>
<td>c.2324T&gt;G*</td>
<td>p.L775X*</td>
<td>46,XX [25]</td>
<td>10.5</td>
<td>82</td>
<td>36.7</td>
<td>46% Elevated for age</td>
<td>yes</td>
<td>no</td>
<td>Alive</td>
<td>(0.6)</td>
</tr>
<tr>
<td>31</td>
<td>F</td>
<td>13</td>
<td>-</td>
<td><em>CBL</em></td>
<td>c.1228 -2A&gt;G</td>
<td>splice site</td>
<td>46,XX [20]</td>
<td>7.4</td>
<td>52</td>
<td>22</td>
<td>7.5</td>
<td>7% Elevated for age</td>
<td>yes</td>
<td>no</td>
<td>Alive</td>
</tr>
<tr>
<td>39</td>
<td>F</td>
<td>26</td>
<td>-</td>
<td><em>CBL</em></td>
<td>c.1111T&gt;C</td>
<td>p.Y371H</td>
<td>46,XX [20]</td>
<td>8.4</td>
<td>46</td>
<td>46</td>
<td>2</td>
<td>10.5% Elevated for age</td>
<td>yes</td>
<td>no</td>
<td>Alive</td>
</tr>
<tr>
<td>55</td>
<td>F</td>
<td>13</td>
<td>-</td>
<td><em>CBL</em></td>
<td>c.1111T&gt;C</td>
<td>p.Y371H</td>
<td>46,XX [20]</td>
<td>8.7</td>
<td>35</td>
<td>50</td>
<td>8.5</td>
<td>1.1% Normal for age</td>
<td>yes</td>
<td>no</td>
<td>Alive</td>
</tr>
</tbody>
</table>

* This mutation has not been reported previously. It is not present in the fibroblasts of the patient.

** This patient displays stable disease rather than spontaneous remission since splenomegaly and cytomorphological abnormalities are still present

M: male, F: female, Hb: haemoglobin; Pl: platelets; WBC: white blood cell count; BM: bone marrow; NA: Not Available