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Germline mutations of the CBL gene define a new genetic syndrome with predisposition to juvenile myelomonocytic leukemia (JMML)

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GERMLINE MUTATIONS OF THE *CBL* GENE DEFINE A NEW GENETIC SYNDROME WITH PREDISPOSION TO JUVENILE MYELOMONOCYTIC LEUKEMIA (JMML)

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ABSTRACT

Background:

CBL missense mutations have recently been associated with juvenile myelomonocytic leukemia (JMML), an aggressive myeloproliferative and myelodysplatic neoplasm of early childhood characterized by excessive macrophage/monocyte proliferation. CBL, an E3 ubiquitin ligase and a multi adaptor protein, controls proliferative signaling networks by downregulating the growth factor receptor signaling cascades in various cell types.

Methods and results:

CBL mutations were screened in 65 patients with JMML. A homozygous mutation of *CBL* was found in leukemic cells of 4/65 (6%) patients. In all cases, copy neutral loss of heterozygosity of the 11q23-qter chromosomal region, encompassing the *CBL* locus, was demonstrated. Three of these 4 patients displayed additional features suggestive of an underlying developmental condition. A heterozygous germline *CBL* p.Y371H substitution was found in each of them and was inherited from the father in one patient. The germline mutation represents the first hit, with somatic loss of heterozygosity being the second hit positively selected in JMML cells. The 3 patients display a variable combination of dysmorphic features, hyperpigmented skin lesions and microcephaly that allow us to tentatively delineate a “CBL syndrome”. Learning difficulties and postnatal growth retardation may be part of the phenotype.

Conclusion:

We report germline mutations of CBL in 3 patients with JMML, confirming the existence of an unreported inheritable condition associated with a predisposition to JMML.

Key words: JMML, CBL, microcephaly, cancer predisposition

INTRODUCTION

The Casitas B-cell lymphoma (CBL, c-CBL) protein is a member of the CBL family of E3 ubiquitin ligases. CBL controls proliferative signaling networks by downregulating the growth factor receptor signaling cascades.[1,2] CBL contains a tyrosine kinase binding (TKB) domain and a Zinc-binding RING finger domain that mediates the E3 ubiquitin ligase activity. These two highly conserved domains are separated by a short linker sequence crucial for ubiquitin ligase activity of CBL.[1] The E3 ligase activity directs the mono-ubiquitination of activated receptors at multiple sites, which promotes endocytosis and lysosomal degradation of the receptors.[2] CBL is also involved in many signaling events through its function as a multiadaptor protein.

CBL missense mutations have recently been associated with acute myeloid leukemia[3,4] various myeloproliferative neoplasms[5,6] and juvenile myelomonocytic leukemia (JMML).[7] JMML is an aggressive myeloproliferative and myelodysplatic neoplasm of early childhood characterized by excessive macrophage/monocyte proliferation that infiltrates hematopoietic and non hematopoietic tissues.[8] The natural course of JMML is rapidly fatal. Progression to acute leukemia is infrequent, but most children die from progressive respiratory and multivisceral failure. Allogenic bone marrow transplantation is the only curative therapy, achieving long term survival in about half of the patients. Cells from affected patients are abnormally sensitive to granulocyte-macrophage colony stimulating factor (GM-CSF). This hypersensitivity is the result of the pathological activation of the RAS signaling pathway by mutations of *NRAS*, *KRAS*, *NF1, PTPN11 or CBL*.[7,8] In JMML, *CBL* mutations were found to target preferentially amino-acid Y371 in the linker region, whereas mutations affecting this amino-acid are rare in other myeloid neoplasms. As in other myeloid malignancies, *CBL* mutations found in JMML are associated with loss of the non mutated *CBL* allele by acquired uniparental disomy of the 11q23 chromosomal region.[5,9]

JMML has been observed in association with congenital malformations, including neurofibromatosis type 1 (NF1) (OMIM 162200) and Noonan syndrome (NS) (OMIM 163950), two conditions associated with RAS pathway deregulation.[8] Patients with NF1 display loss of the normal *NF1* allele in cancer cells.[10] Patient with NS and

JMML usually harbor activating heterozygous germline mutations in *PTPN11*[11] and more rarely, in *KRAS*[12], or *NRAS*[13].

Germline *CBL* mutation was suspected in one patient by Loh et al.[7] who detected a heterozygous p.Y371H CBL mutation in cord blood and a homozygous p.Y371H mutation in JMML cells. We confirm here that some patients with JMML can harbor germline mutations of CBL by reporting 3 further patients among a cohort of 65 JMML patients. Our observations suggest that constitutional *CBL* mutations lead to a constellation of mild, variable developmental defects and predisposition to JMML, which could be tentatively described as a "CBL syndrome".

PATIENTS / METHODS

Patient 1

General history

Patient 1 was born at 38 weeks of gestation (WG). Her mother had gestational diabetes. Birth weight (BW) was 3020 g (-0.6 SD), birth length (BL) 45 cm (-2.4 SD), occipito-frontal head circumference (OFC) 32 cm (-2.2 SD). She had failure to thrive, with poor sucking and postnatal growth retardation. She walked at the age of 24 months (mo). She uttered her first words at 23 mo. Subsequent development of speech was severely delayed. She had normal hearing and normal ophthalmologic examination. She developed a JMML at age 26 mo. At first evaluation, in the genetic department of Nantes, at age 30 mo, she was 78 cm tall (-3 SD), had a weight of 9.6 kg (-2.1 SD) an OFC of 44.5 cm (-2.7 SD). She was felt to be mildly dysmorphic, disclosing broad forehead, hypertelorism, epicanthic folds, deeply grooved philtrum, thick lips, mild retrognathism, thick, posteriorly rotated but normally set ears with overfolded helices, short neck, thin hair and low posterior hairline (Figure 1 A-B). She had a single café-au-lait spot on the abdomen. She had no thoracic or spinal deformities. The cardiac ultrasound scan was normal. The cerebral MRI was normal. She was hyperactive, with short attention span and poor verbal skills. At that time, a diagnosis of mild Noonan syndrome was tentatively suggested. At last evaluation,

aged four years (y) six mo, her height was 96 cm (-1.7 SD), her weight was 13.7 kg (-1.3 SD) and her OFC is 47 cm (-2.2 SD).

Family history

This girl was the second child of healthy non consanguineous parents aged 27y and 22y at time of her birth. The father was 180 cm tall (+ 0.8 SD). He has no personal or familial history of hematologic malignancies. He had a vocational training qualification. The mother was 155 cm (- 1.5 SD). Several relatives of the mother had adult-onset cancers of various types.

Hematological history

At age 26 mo, she was hospitalized for massive hepatosplenomegaly, retroperitoneal and mesenteric lymphadenopathies, hyperleucocytosis ($46x10^9$/L), thrombocytopenia ($46x10^9$/L) and anemia (8.4 g/dl). The peripheral differential blood count showed: 17% monocytes ($7.6x10^9$/L), 18% immature granulocytes and 3% blasts. The bone marrow aspirate was hypercellular with granulocytic proliferation but without excess of blasts. These features were consistent with a diagnosis of JMML. She received cord blood, then relapsed and benefited from a second cord blood allograft. She is alive, 16 months after the second allograft and displays complete donor chimerism.

Patient 2

General history

Patient 2 was born in Tunisia at 41 WG by caesarean section for dystocia. Birth Weight: 3100 g (-0.4 SD), birth length 50 cm (-0.9 SD), OFC 34 cm (-1 SD). The pregnancy was uneventful. She had postnatal failure to thrive. At age 1 year, her height was 71 cm (-0.6 SD), her weight was 8 kg (-1.2 SD) and her OFC is 44 cm (-1.1 SD). At last evaluation, at age 24 mo she weighted 8 kg (-3 SD), her height was 77 cm (-2.4 SD) and her OFC was 44.5 cm (-2.4 SD). She had microcephaly, triangular facies, high cranial vault, bilateral epicanthic folds, thick lips, prominent philtrum, posteriorly rotated helices and somewhat sparse hair (Figure 1 C-D). The cardiac echocardiogram was normal. She has no skin anomalies. T2-weighted Flair MRI showed non-specific hyperintense signals in the periventricular white matter. Neurological examination was normal. Psychomotor development was appropriate

for age (walked at 18 months, first words at 12 months). Bone age was mildly delayed (18 mo at age 22 mo). IGF1 was 12 nmol/l (normal 4 to 22 nmol/l) and IGFBP-3 1100 ng/ml (normal 1090 to 2490 ng/ml).

Family history

She was the only child from healthy first cousins parents. The mother was 26y old and the father 39y old at birth. The father was 168 cm tall (- 0.6 SD) and the mother 160 cm (- 1.2 SD). The family history was not contributory.

Hematological history

A diagnosis of JMML was made at 13 months of age because of persistent hyperleucocytosis ($50x10^9$/L), thrombocytopenia (35.10^9/L) and hepatosplenomegaly. The peripheral differential blood count showed: 19% monocytes ($9.5x10^9$/L), 9% immature granulocytes and 0.5% blasts. The bone marrow was hypercellular with granulocytic proliferation and no excess of blasts. At age 24 months, she received a cord blood allograft. She remains alive at age 25 months. A mixed chimerism (50% donor) was found 30 days after the allograft.

Patient 3

General history

Patient 3 was born at 39 WG. Her mother had gestational diabetes. BW: 3240g (-0.1 SD), BL 50 cm (+ 0.3 SD), OFC 35 cm (+0.55 SD). At age 12 mo, she was 70.5 cm tall (-0.8 SD), and weighted 8.8 kg (-0.4 SD) and her OFC was 45 cm (-0.25 SD). She had a broad forehead, arched eyebrows, hypertelorism, palpebral ptosis, short, upturned nose, flat malar areas, deeply grooved philtrum, posteriorly rotated but normally set ears with thick helices and large lobules (Figure 1 E-F). Pectus excavatum and hypermobile finger joints were present. Her skin was hyperelastic and she presented redundant skinfolds on hands and feet. Three café-au-lait spots (smaller than 2 cm) were present on the back and two on the anterior part of the thighs. The cardiac ultrasound and cerebral MRI were normal. Neurological, ophthalmologic examinations and hearing test were normal. She walked unsupported at age 14 mo and uttered her first words at 18 mo. She displayed a postnatal decline of her OFC (at age 2 years her OFC was 46 cm (-1.2 SD) and at age 3 years 47.5 cm

(-1.1 SD)). At last evaluation, aged six years, her height was 107 cm (-1.4 SD) and her weight was 15 kg (-1.9 SD). She had normal schooling (first grade) and no learning disabilities.

Family history

This girl was the only child of healthy non consanguineous parents aged 27y and 23y at the time of her birth. The father was 193 cm tall (+3.0 SD) and the mother 153 cm (-1.8 SD). The family history was not contributory.

Hematological history

At age 12 mo, she was hospitalized for massive splenomegaly, hyperleucocytosis ($30x10^9$/L), mild thrombocytopenia ($136x10^9$/L) and anemia (9.4 g/dl). The peripheral differential blood count showed: 22% monocytes ($6.6x10^9$/L), 5% immature granulocytes and 1% blast. The bone marrow aspirate was hypercellular with granulocytic proliferation but without excess of blasts. She had a cord blood allograft. She had no relapse 4 years and 9 months after the allograft. She has complete donor chimerism.

Genetic analysis

Parents gave written consent for genetic analysis and clinical photographs.

Karyotyping

Conventional cytogenetic analysis was performed on peripheral blood and bone marrow using standard procedures.

Mutation screening

Bone marrow aspirates and peripheral blood were collected on EDTA at diagnosis. Genomic DNA was extracted from mononucleated hematopoietic cells, fibroblasts or buccal swabs using Qiagen Mini or Midi Kit (Qiagen Ltd). Mutation screening was performed by bi-directional sequencing of exons and their flanking intron-exon boundaries as described previously.[14] The entire coding region of *KRAS* and *NRAS* was screened. *PTPN11* screening was restricted to exons 2, 3, 4, 7, 8, 12, 13, 14 and *CBL* screening to exons 7, 8 and 9. GenBank accession number for *CBL*

genomic and mRNA reference sequences are NM_005188 and NC_000011 respectively.

Microsatellite analysis

Loss of heterozygosity (LOH) at *CBL* locus was assessed by PCR amplification of 9 microsatellite markers covering the 11q arm: D11S1294 (11q22.3), D11S4206 (11q22.3), D11S4129 (11q22.3), D11S924 (11q22.3), D11S4171 (11q22.3), D11S1774 (11q22.3), D11S925 (11q23), D11S934 (11q23-24), D11S968 (11q25).

LOH at *NF1* locus was assessed by PCR amplification of 8 microsatellite markers covering the 17q arm located within or close to the *NF1* gene respectively.[15]

Family links were checked by testing a panel of 16 microsatellite using the powerplex® kit (Promega).

SNP array analysis

Tumor (bone marrow cells) and fibroblasts DNA were hybridized to Affymetrix Genome-Wide Human SNP 6.0 Arrays. CEL files were created using the Affymetrix GeneChip Command Console operating software and Genotyping Console 2.1, according to the manufacturer's protocols (Affymetrix). The Partek Genomics Suite was used for both copy number alteration (CNA) and LOH analysis. Regions of CNA were detected using a Hidden Markov Model algorithm in the standard Partek workflow for paired samples. LOH used genotyping results from paired germinal and tumor samples and Partek LOH workflow.

Myeloid progenitor cell growth

In vitro growth of myeloid progenitors was performed by plating bone marrow and peripheral blood mononucleated cells in semi-solid methylcellulose with and without leukocyte conditioned medium (cytokines medium, LCM, StemCell Technologies Inc, Vancouver, Canada) as described previously.[16] Colonies (aggregates containing >50 cells) were scored at day 11 and 14. “Endogenous growth” corresponded to the presence of CFU (CFU-GM>10 colonies or CFU-M>5 colonies) in absence of growth factors.

RESULTS

CBL mutations were screened in a cohort of 65 unselected patients with JMML. A homozygous mutation of *CBL* was found in leukemia cells of 4/65 (6%) patients. In all patients, copy neutral loss of heterozygosity of the 11q23-qter chromosomal region, encompassing the *CBL* locus, was demonstrated by SNP array (Figure 2) and microsatellite analysis. No RAS activating mutation in *NRAS*, *KRAS*, or *PTPN11* gene (classically associated with JMML) was detected. None of the patients had phenotypic feature evocative of NF1, except for some café-au-lait spots in patients 1 and 3. Mitotic recombination leading to loss of heterozygosity and subsequent inactivation of both *NF1* alleles is virtually constant in JMML of patients with neurofibromatosis.[10] No loss of heterozygosity at the *NF1* locus was present in the leukemia cells of our 3 patients, making unlikely the presence of a NF1 in these children.

One JMML patient had a c.1254C>G homozygous transversion in exon 9 of *CBL*, resulting in the p.F418L missense amino acid substitution. The three others had a c.1111T>C homozygous transition (in exon 8), resulting in the p.Y371H missense amino acid substitution (Figure 3.1). These patients displayed additional features suggestive of an underlying developmental condition. All three had normal blood and bone marrow karyotype. A heterozygous germline p.Y371H substitution was found in fibroblasts in each of them (Figure 3.2). DNA of jugal mucosa (obtained from buccal swabs) was tested in patients 2 and 3 and also showed the presence of the germline p.Y371H substitution. In patient 1, despite a family history of cancer in maternal lineage, the mutation was inherited from the father (Figure 3.4). Mutation occurred *de novo* in the 2 other patients (Figure 3.4).

These three patients fulfilled all the JMML diagnosis criteria reported at the last JMML international symposium[17] and their presentation did not significantly differ from that observed in the other patients (age at diagnosis, WBC count…). Myeloid progenitors from blood and bone marrow showed an endogenous growth pattern in the absence of growth factors, as classically observed in JMML (data not shown).

The three children present slightly dysmorphic traits. Two patients have multiple café-au-lait spots. Two have microcephaly below -2 SD. Patient 1 had short birth length, and secondary catch up ending in low normal stature at age 4.5 years. Patient 2 had

true growth decline during her second year of life, ending with true postnatal growth retardation. No growth retardation was noted in patient 3 and in the father of patient 1.

DISCUSSION

We identified constitutional heterozygous missense mutations of *CBL* in three unrelated patients with JMML, confirming the existence of a currently unrecognized dominantly inherited condition with propensity to develop myeloproliferative neoplasm (JMML) in early infancy. Our patients carried the recurrent p.Y371H substitution, inherited from the father in one instance. The process of tumorigenesis observed in our patients is in line with the classical Knudson hypothesis for tumor suppressor genes: the first hit is germline while the second hit occurs somatically and is selected for in JMML cells. Phosphorylation of Y371 is essential for the E3 activity of CBL and for its interaction with a number of signaling proteins. Phospho-Tyr-371 appears to play a role in the interactions of c-Cbl with PI3 kinase (PI3K).[2] Substitution of Y371 can have different consequences: mutants of CBL lacking Y371 are oncogenic,[18] Y371F mutants are inactive in auto-ubiquitylation assays *in vitro*, while Y371E mutants apparently mimic phosphorylated tyrosines and are constitutively active.[2]

Although our series is small, we found subtle developmental anomalies in all patients. Mild hypertelorism, short upturned nose, deeply grooved philtrum and thick lips, reminiscent of the facial Gestalt of Noonan syndrome, are observed in the 3 patients. Two patients have café-au-lait spots. Patient 1 had short stature at birth and low normal height at last evaluation, whereas patient 2 showed progressive growth retardation. These two children have mild microcephaly, present at birth in one of them. In patient 1, developmental milestones are delayed. For patient 2, there is no major developmental anomaly, but her young age precludes any firm conclusion. Patient 3 had a normal head circumference at birth and presented a postnatal decline of her OFC. She had a normal development, as did patient 1's father. Although these anomalies could have occurred by chance, presence of some dysmorphic signs in each patient lead us to raise the hypothesis that *CBL* haploinsufficiency may interfere

with normal somatic and cerebral development. Unfortunately, no phenotypic data are available for the only other patient with constitutional *CBL* mutation reported by Loh et al.[7] At this point, these somewhat conflicting observations do not allow us to make a firm conclusion, considering the possible confounding effects of JMML development itself, and of therapeutic and nutritional interventions. The dysmorphic traits are subtle, and need to be confirmed by independent reports and long term follow-up, but the fact that Noonan syndrome was discussed in case 1 prior to the discovery of the CBL anomaly has to be underlined. Time will tell if our seminal observations represent the first step in the delineation of a "CBL syndrome".

RAS activation is known to play a central role in sporadic and syndromic JMML. *CBL* germline mutations are associated with a propensity to develop JMML suggesting that they induce RAS pathway activation. However, they are associated with a phenotype that is distinct from Noonan syndrome, neurofibromatosis, or any other disorder belonging to the spectrum of RAS/MAPK activation syndrome. Significantly, congenital heart defect were not observed in our patients.

Abnormalities in the ubiquitylation system have been implicated in the pathogenesis of various human disease including malignant transformation and several genetic diseases.[19] Knock-out of either the Cbl or Cbl-b genes in mice are mainly characterized by a phenotype in the lymphocyte compartment.[20,21] However, $Cbl^{-/-}$ mice also display a relatively mild phenotype in other cell types. In addition, recent data suggest that mutations do not lead to the simple knock-out of Cbl function but render it a proto-oncogene.[22] Mutant CBL inhibits ubiquitination of growth factor receptors even in the presence of a normal copy of the *CBL* gene, leading to enhanced proliferative response to growth factors.[6,22] Altogether, these observations are consistent with a phenotypic expression of CBL heterozygous mutation.

The importance of Cbl in hematopoiesis has been demonstrated in knock-out mice that show hyper-responsiveness to hematopoietic growth factors, expansion of the progenitor and stem cell pool, and mild myeloproliferative features.[22] The observation of a propensity to develop JMML in children with CBL germline mutations as well as the close association of CBL mutations with other monocyte expansion, such as that seen in CMML or in AML with monocytoid features suggest a primary role for CBL mutations in the pathogenesis of these disease. The reason for the specific impact of CBL on the monocytic lineage and the role of RAS in this process has to be clarified.

Intriguingly, Chiusaroli et al. reported that growth is transiently delayed in $Cbl^{-/-}$ mice.[23] This could be explained by a delayed replacement of cartilage by bone as a consequence of a decreased motility of osteoclasts. CBL inactivation may also interfere with growth hormone (GH) signalling.[24]

How can we understand the presence of microcephaly in 2/3 patients? Dysregulation of neuroepithelial progenitors proliferation and survival is suspected to be a major cause of primary microcephaly.[25] Loss of CBL function, by contributing to the activation of the JNK pathway, may sensitize healthy neuronal cells to death.[26] It has been recently shown that deficiency of the small GTPase Rac-1 in mouse forebrain causes microcephaly by increasing apoptosis and defective differentiation.[27] Thus, decreased activation of RAC-1 by CBL may also play a role by inducing a pro-death signaling cascade.[2]

In conclusion, presence of germline alteration of CBL yields a relatively mild phenotype in childhood, possibly because of partial redundancy of CBL and CBL-b functions. However, it raises several concerns for the follow-up of those patients. Inappropriate activation of mammalian protein tyrosine kinases (PTK) can lead to various forms of human cancers. Could CBL mutation predispose to other malignancies, or to secondary solid tumors in JMML survivors? Which prevention strategy should be proposed?

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FIGURE LEGENDS

Figure 1: Photographs of the three patients.

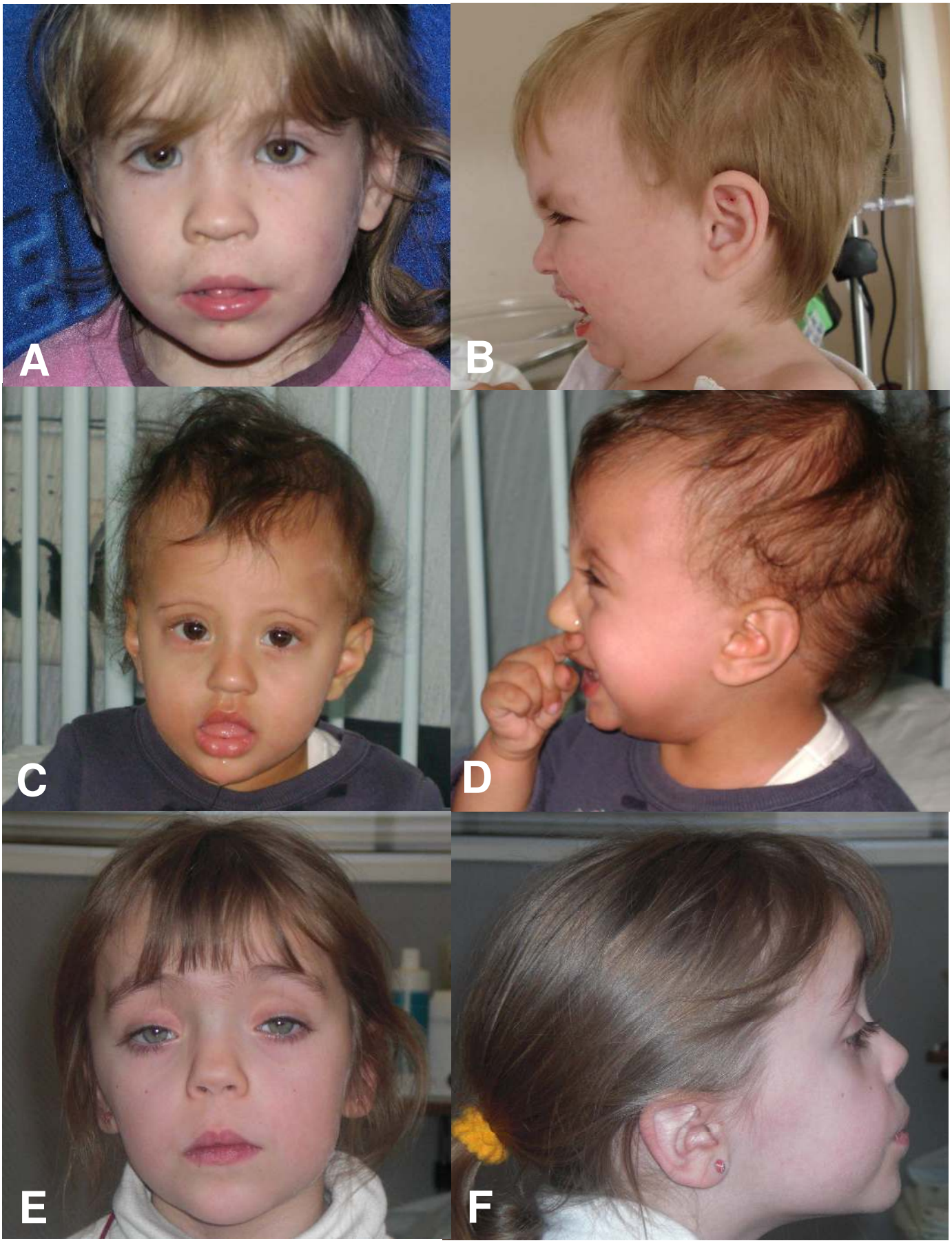
A, C, E: frontal facial view of patient 1 (at 4 years 6 months of age), of patient 2 (at 24 months of age), of patient 3 (at 6 years of age).

B, D, F: lateral facial view of patient 1 (at 30 months of age), of patient 2 (at 24 months of age), of patient 3 (at 6 years of age).

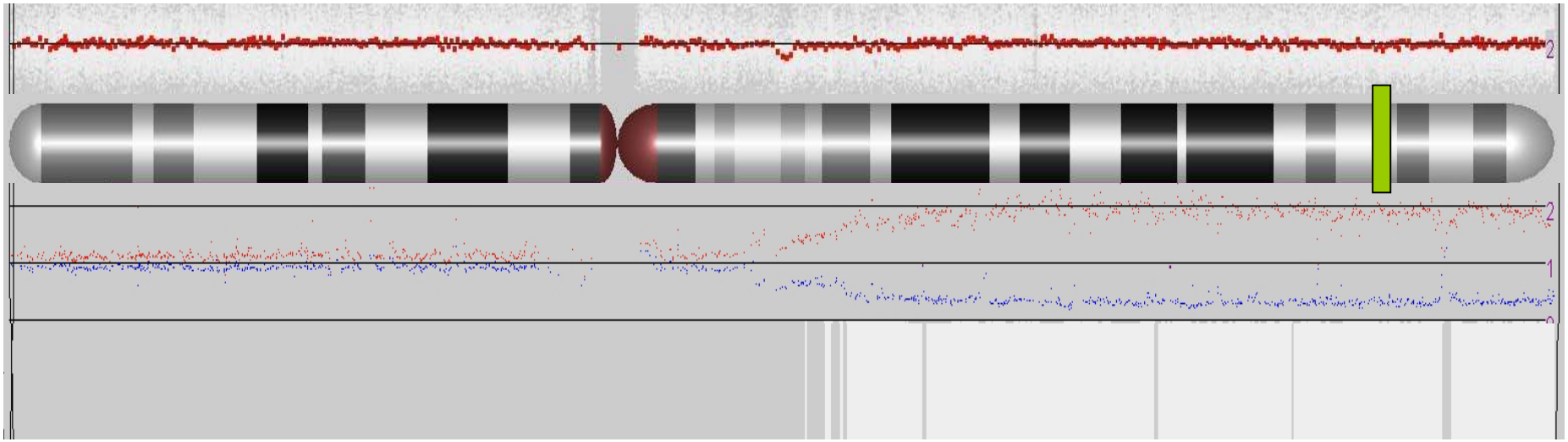
Figure 2: Single nucleotide polymorphism array profile of chromosome 11 (Affymetrix SNP6.0 technology and Partek image) showing the copy-neutral LOH of tumor versus germinal DNA for patient 1. The red line represents average copy number signal intensity of SNPs on the array chip. In this instance, there are no CN variations and the red line does not deviate from normal diploid CN. The graph above chromosome ideogram represents the allele specific copy number and indicates copy-neutral LOH. White box represent UPD region.

Figure 3: Germline *CBL* mutations. Sequence electrophoregrams documenting the c.1111T>C mutation found in exon 8 of *CBL* gene for these three patients:

3.1, DNA at time of the leukemia diagnosis; 3.2, DNA from cultured fibroblasts; 3.3, maternal DNA; 3.4, paternal DNA. Asterisks (*) indicate homozygous state, crosses (x) indicate heterozygous state.

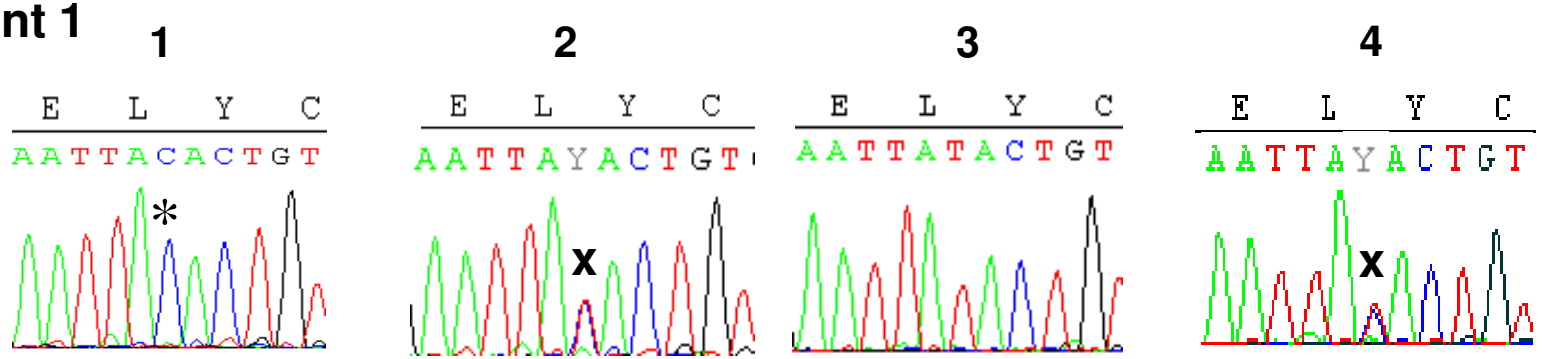

A
B
C
D
E
F

CBL 11q23

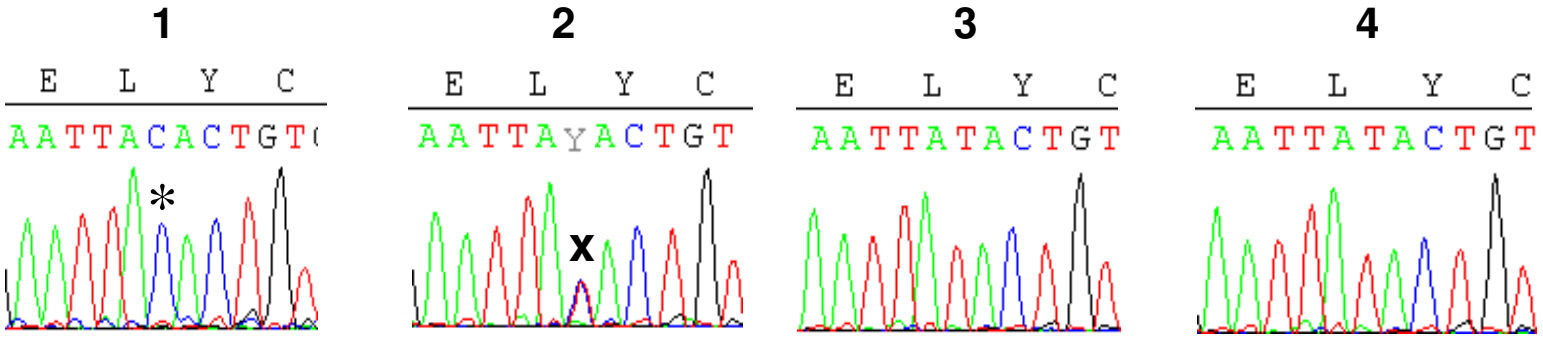


Chromosome 11

Patient 1



Patient 2



Patient 3