Two novel mutations in the BCKDK gene (branched-chain keto-acid dehydrogenase kinase) are responsible of a neurobehavioral deficit in two pediatric unrelated patients

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ABSTRACT

Inactivating mutations in the BCKDK gene, which codes for the kinase responsible for the negative regulation of the branched-chain keto-acid dehydrogenase complex (BCKD), have recently been associated with a form of autism in three families. In this work, two novel exonic BCKDK mutations, c.520C>G/p.R174G and c.1166T>C/p.L389P, were identified at the homozygous state in two unrelated children with persistently reduced body fluid levels of branched-chain amino acids (BCAAs), developmental delay, microcephaly and neurobehavioral abnormalities. Functional analysis of the mutations confirmed the missense character of the c.1166T>C change and showed a splicing defect r.[520c>g;521_543del]/p.R174Gfs1*, for c.520C>G due to the presence of a new donor splice site. Mutation p.L389P showed total loss of kinase activity. Moreover, patient-derived fibroblasts showed undetectable (p.R174Gfs1*) or barely detectable (p.L389P) levels of BCKDK protein and its phosphorylated substrate (phospho-E1α), resulting in increased BCKD activity and the very rapid BCAA catabolism manifested by the patients’ clinical phenotype. Based on these results, a protein-rich diet plus oral BCAA supplementation was implemented in the patient homozygous for p.R174Gfs1*. This treatment normalized plasma BCAA levels and improved growth, developmental and behavioral variables. Our results demonstrate that BCKDK mutations can result in neurobehavioral deficits in humans and support the rationale for dietary intervention.

Key words: BCKDK; neurobehavioral deficit; dietary treatment; branched-chain keto-acid dehydrogenase complex
INTRODUCTION

Mutations in the *BCKDK* (branched-chain keto-acid dehydrogenase kinase) gene (MIM# 614901) have recently been described as a cause of comorbid intellectual disability, autism and epilepsy in three consanguineous families (Novarino et al., 2012). These patients also showed abnormally low plasma levels of branched-chain amino acids (BCAAs).

BCAAs cannot be stored in any form other than protein; excess of BCAAs must therefore be removed. The BCAA catabolic pathway includes a deamination step followed by the irreversible oxidative decarboxylation of the deaminated products - branched-chain α-keto acids (BCKAs) – to their corresponding acyl-CoA esters. This is catalyzed by mitochondrial branched-chain amino acid amino transferase and the branched-chain α-keto acid dehydrogenase complex (BCKD) (Islam et al., 2010). Human BCKD is a macromolecular machine composed of four catalytic subunits encoded by four different genes: E1α-*BCKDHA* (MIM# 608348), E1β-*BCKDHB* (MIM# 248610), E2-*DBT* (MIM# 248610) and E3-*DLD* (MIM# 238331) (Chuang and Shih, 2001). These four subunits are assembled to form a mitochondrially-located complex that shows strong structural homology to the pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase complexes. In fact, they share the same E3 subunit. The complex occupies a strategic point in the BCAA catabolic pathway, and a careful regulation of its activity is crucial in correct BCAA metabolism (Harris et al., 2005). As seen with PDH, the overall activity of the BCKD complex is controlled by the phosphorylation (inactivation)/dephosphorylation cycle and is influenced by dietary and hormonal stimuli (Reed et al., 1985). The molecular mechanism for the acute inactivation of the BCKD complex involves the phosphorylation of a serine residue at position 293 in the E1α subunit, a step catalyzed by a specific mitochondrial BCKD kinase bound to the E2 subunit (Li et al., 2004; Wynn et al., 2004). Mitochondrial BCKDK plus the pyruvate dehydrogenase kinases (PDKs) form a well characterized family of protein kinases - the
mitochondrial protein kinases (Davie et al., 1995). Recombinant rat BCKDK expressed in *E. coli* as a bacterial maltose-binding fusion protein (MALBP-BCKDK) has been used to resolve its atomic structure (Machius et al., 2001) and to measure its kinase activity (phosphorylation of the BCKD subunit E1 alpha and autophosphorylation) (Wynn et al., 2000). The structure of BCKDK features a characteristic nucleotide-binding domain plus a four-helix bundle domain reminiscent of modules found in protein histidine kinases (Machius et al., 2001) that are involved in two-component signal transduction systems. BCKDK is also the subject of allosteric regulation by BCKA levels and other factors (Huang and Chuang, 1999). BCKAs inhibit BCKDK and attenuate its interaction with the BCKD complex (Harris et al., 1994, 2004). The unphosphorylated active state is regained via the activity of a mitochondrial protein phosphatase (PP2Cm), encoded by the *PPM1K* gene - a critical regulator of BCAA catabolism in mice and humans (Joshi et al., 2007; Lu et al., 2009; Wynn et al., 2012; Zhou et al., 2012; Oyarzabal et al., 2013). When BCAA levels are low, or there is a need to conserve BCAAs for protein synthesis, BCKD is shifted towards its inactive form, leading to the accumulation of BCAAs. The phosphorylation state of the complex correlates inversely with the level of BCKDK. The recent identification of patients harboring mutations in the *PPM1K* (Oyarzabal et al., 2013) or *BCKDK* gene (Novarino et al., 2012) highlighted the importance of careful regulation in the oxidative disposal of BCAAs for normal human development.

Here we report two new, unrelated patients with BCKDK deficiency, both showing a neurobehavioral deficit associated with low concentrations of BCAAs in their body fluids. The mutations identified in the *BCKDK* gene responsible for their conditions are described. We also show that the provision of a high-protein diet supplemented with BCCAs normalizes plasma BCAAs levels and improves neurological symptoms.

**MATERIAL AND METHODS**
Patients

The present patients were referred from different clinical centers. Supp. Table S1 shows their clinical examination findings, neurodevelopment scale scores, and electroencephalography (EEG) and brain magnetic resonance imaging (MRI) results. Samples from patients were obtained in accordance with the Helsinki Declaration of 1964, as revised in 2000. Ethical approval for the use of patient samples was granted by the institutional Ethics Committees of the Hospital Sant Joan de Déu and the Universidad Autónoma de Madrid.

Patient 1 (P1) was a boy, the fourth child of consanguineous (third degree) Arab parents. His three older siblings (two girls and one boy) are all healthy. Pregnancy and delivery were normal. His birth weight was 3.310 kg (-0.5 SD), head circumference 33 cm (-2 SD), and Apgar test score 9/10/10. Psychomotor delay, gastrointestinal symptoms (such as frequent vomiting with no particular trigger) and moderate anorexia were present since the first months of life. The patient was referred to a hospital at 18 months of age. Physical examination performed at the hospital recorded the following: weight 9.4 kg (-1 SD), height 74 cm (-2 SD), head circumference 44.5 cm (-2.5 SD), no dysmorphic features, no skin lesions, and no liver or spleen enlargement. Severe developmental delay (including the inability to walk) and behavioral abnormalities were the most striking neurological signs (Supp. Table S1).

Patient 2 (P2) was a boy, the second child of non-consanguineous Spanish parents. His older brother is healthy. Pregnancy and delivery were normal. His birth weight was 3.250 Kg (-0.5 SD) and Apgar test score 9/10/10. Psychomotor delay was present from the first months of life; he did not walk until 22 months of age. He was referred to a hospital for developmental delay at 2 years of age. Physical examination performed at the hospital recorded a short neck, bulbous nose and short distal phalanges. No skin lesions were detected, nor was the liver or spleen enlarged; his weight was 12.5 kg (0 SD), his height 84 cm (-0.5 SD), and his head circumference 44.5 cm (-2.5 SD).
circumference 46 cm (-2.5 SD). Developmental delay and behavioral abnormalities were again the most striking neurological signs (Supp. Table S1).

**Molecular genetics**

Genomic DNA and total mRNA were isolated from blood samples or primary cultured fibroblasts by standard procedures using the MagnaPure system (Roche Applied Science, Indianapolis, USA).

Sanger and whole exome sequencing were used to detect mutations in the **BCKDK** gene (RefSeq accession number NG_033011.1).

**Sanger sequencing**

Coding region and flanking intron-exon boundaries were PCR amplified with primers based on the Ensemble genome browser entry ENSG00000103507. All PCR products were sequenced using BigDye Terminator v.3.1 Mix (Applied Biosystem Foster City, CA, USA) and analyzed by capillary electrophoresis using an ABI Prism 3700 Genetic Analyzer (Applied Biosystems). DNA mutation numbering was based on cDNA reference sequence (NM_005881.3), taking nucleotide +1 as the A of the ATG translation initiation codon. The mutation nomenclature used follows that described at http://www.hgvs.org./mutnomen/. The **BCKDK** variations have been submitted to the locus-specific database (www.lovd.nl/BCKDK).

For the full length open reading frame amplification by RT-PCR, total RNA obtained as described above, was retro-transcribed using the SuperScript® VILOTM cDNA Synthesis Kit (Life Technologies). **BCKDK** cDNA was amplified using the FastStart Taq Polymerase kit (Roche).
The DNA of the patients’ relatives, and that of 300 European control subjects (Human Random Control DNA Panel-1 HRC-1 [ECAA and Sigma-Aldrich]), was tested for the detected mutations by bidirectional sequencing or high-resolution melting analysis (Richard et al., 2009), using LightCycler 480 High Resolution Melting Master and apparatus (Roche Applied Sciences).

**Exome sequencing**

Base calling and quality control were performed using the Illumina RTA sequence analysis pipeline. Analysis of the primary data (FASTQ files) was done using the BIER’s platform pipeline (CIBERER) which was as follows. Sequence reads were aligned to the human reference genome build GRCh37 (hg19) using the Burrows-Wheeler Aligner (BWA) (Li and Durbin, 2009). Mapped reads were filtered (leaving only those mapping in unique genomic positions with enough quality), sorted and indexed with SAMtools (Li et al., 2009). GATK (McKenna et al., 2010) was then used to realign the reads as well as for the base quality score recalibration. Once a satisfactory alignment was achieved, identification of single nucleotide variants and Indels was performed using GATK standard hard filtering parameters (DePristo et al., 2011). For the final exome sequencing analysis report we used the VARIANT (Medina et al., 2012) annotation tool, which provide additional relevant variant information for the final process of candidate gene selection. In particular, minor allele frequency (MAF), obtained from dbSNP (Sherry et al., 2001) and 1000 Genomes project (Abecasis et al., 2012), was provided to help on the selection of undescribed variants in healthy population. SIFT (Kumar et al., 2009) and Polyphen (Adzhubei et al., 2010) damage scores were computed to predict the putative impact of the discovered variants over the protein structure and functionality. This information was completed with data on evolutionary conservation obtained from PhastCons (Siepel et al., 2005) along with disease related annotations, at both variant and gene levels. Finally, GO terms for the affected genes were also retrieved. The
successive application of quality control filters and the prioritization by the parameters accounting for potential functional impact led us to build up a list of candidate genes (and variants) ranked by its segregation with the cases and the putative potential impact. Such prioritization list was further inspected to look for potential candidate genes and/or variants.

**In silico predictions**

Potential 3´and 5´splice sites in the sequence covering positions c.424_543 of the human \textit{BCKDK} gene were analyzed using the NNSplice 0.9 program (http://www.fruitfly.org/seq_tools/splice.html) and the Human Splicing Finder 2.4.1.(http://www.umd.be/HSF/).

**Cell culture**

Dermal fibroblasts from the patients were grown from a skin biopsy (taken with informed consent) following standard conditions in minimal essential medium (MEM) supplemented with 1% glutamine, 10% foetal calf serum (FCS) and antibiotics. These dermal fibroblasts were immortalized using pBABE-puro containing SV40 DNA sequences (courtesy of Dr. J. A. Enriquez). Control cell lines GM08680 (Coriell Institute for Medical Research, NIGMS Human Genetic Cell Repository, USA), and human dermal fibroblasts CC2509 (Lonza, Spain) were used.

**Western blot analysis**

Western blot analysis of the mitochondrial fraction from patient and control fibroblasts, isolated by magnetic separation using MACS\textsuperscript{®} Technology, was performed as described in Supplementary Methods. The primary polyclonal antibodies used were those against total BCKDE1-alpha (ab68094, Abcam), BCKD kinase (ab111716, Abcam), the phosphor-S293 BCKDE1-alpha epitope (A303-567A, Bethyl), and Hsp60 (Stressgen, MI, USA). To validate
the specificity of recognition for the phosphor-S293 BCKDE1-alpha epitope by its cognate anti-Phospho BCKDE1 alpha (S293), we used a Phospho Blocking Peptide (Bethyl Laboratories) that disrupts antigen-antibody interactions. Maximal disruption was achieved by incubating 4 μg of anti-Phospho BCKDE1 alpha (S293) with 20 μg of Phospho Blocking Peptide before performing western blot analysis.

**Lentiviral constructs**

The full-length ORF of human BCKDK was cloned into the mammalian lentiviral plasmid pEZ-Lv205 (EX-M0284-Lv205), which contains an eGFP reporter gene with an IRES sequence (GeneCopoeia, Rockville, MD, USA). The same vector backbone, but containing only the eGFP gene, was used as a transduction control. Lentiviral stock production and fibroblast infection were performed as described elsewhere (Bovia et al., 2003; Richard et al., 2013). Efficiently infected fibroblasts were sorted using a FACSVantage Turbo Sorter (BD Biosciences).

**Biochemical analysis**

Branched-chain amino acids were quantified by ion-exchange chromatography, and BCKAs in plasma by GC-MS essentially as previously described (Chalmers and Lawson, 1982).

**Stable isotope tracer-based metabolite analysis**

Culture medium (BCAA-free MEM, supplemented with 0.8 mmol/L of [U-13C] leucine (Cambridge Isotope Laboratories, Inc.) [MEM-Leu]) (Bixel et al., 2004), from flasks in which patient and control fibroblasts were grown for 24 h was sampled for metabolite profiling by HPLC-MS/MS. See Supplementary Methods for a detailed description.

**Recombinant MALBP-BCKDK (wild-type and mutant) kinase activity**
The kinase activity of the identified mutants was analyzed using the fusion protein MALBP-BCKDK expressed in \textit{E. coli}, and following a protocol similar to that described for rat BCKDK (Davie et al., 1995). See Supplementary Methods for a detailed description.

\section*{RESULTS}

\textbf{Biochemical analysis: metabolite signature for dysfunction in BCAA catabolism}

Low levels of BCAAs were recorded in both patients’ plasma, cerebrospinal fluid (CSF) and urine. Plasma BCKA levels were also low (Fig. 1 A). Stable isotope tracer-based analysis of MEM-Leu medium in which fibroblasts had been grown for 24 h showed a significant reduction in [\text{U-^{13}C}] \alpha-keto-isocaproate compared to that in which control cells were grown (Fig. 1 B). These results indicate an increased rate of BCAA catabolism in these fibroblasts, compatible with a constitutively active BCKD complex.

\textbf{Genetic analysis}

To identify the mutations underlying the metabolic disorder in these patients, whole-exome sequencing, plus further confirmatory Sanger sequencing of the \textit{BCKDK} gene for P1 and direct Sanger sequencing of the \textit{BCKDK} gene for P2, was performed. Two novel, homozygous \textit{BCKDK} nucleotide changes were identified: c.520C>G in P1; and c.1166T>C in P2 (Fig. 2 A). Both mutations were absent from 300 control alleles, and segregated in the families as a recessive trait. The predictable effects of these sequence variations on the protein might be a p.R174G and p.L389P change respectively. However, when the corresponding mRNAs were examined, the results did not agree with the first of these predictions (Fig. 2 B-C). For c.520C>G, RT-PCR and sequencing showed a unique mutant transcript with a 23 bp deletion resulting from the creation of a new donor splice site within exon 6 in P1-derived fibroblasts. The product of this mutant transcript, r.[520c>g; 521_543del], led to a frameshift creating a premature stop codon (p. R174Gfs1*). However,
the presence of some wild-type spliced protein product (p.R174G) cannot be ruled out in the organs of P1. The intrinsic activity of the P1 missense mutation was therefore studied.

Functional effect of BCKDK mutations

The impact of the identified missense mutations on BCKDK activity was studied using purified recombinant human proteins. Wild-type BCKDK showed autophosphorylation and phosphorylated its natural substrate (subunit E1α of BCKDH complex) (Fig. 3), as well as the generic kinase’s substrate Myelin Basic Protein (MBP) (Supp. Figure S1). No residual activity was seen for mutant L389P, whereas mutant R174G conserved >50% of the wild-type protein activity (Fig. 3 and Supp. Figure S1).

The functional consequences of the BCKDK p.L389P and BCKDK p.R174Gfs1* mutations were analyzed in cell-based experiments, using mitochondrial extracts from immortalized patient fibroblasts. Severely reduced/undetectable levels of the BCKDK protein mutants were recorded, along with a total absence of phospho-S293 BCKDE1-alpha. However, levels were recovered by lentiviral transduction of eGFP-tagged wild-type BCKDK (Fig. 4 A). The presence of wild-type BCKDK in the mitochondria was assessed by immunofluorescence microscopy using anti-BCKDK antibodies (Fig. 4 A). A normal phospho-S293 BCKDE1-alpha status was detected in the cell lines of both patients after harvesting the mitochondrial protein from transduced cells. No evidence of increased levels of BCKDK protein or phospho-S293 BCKDE1-alpha was seen in mock-infected cells (Fig. 4 B). To check the specificity of recognition for the phosphor-S293 BCKDE1-alpha epitope by anti-phospho-S293 BCKDE1-alpha antibody, the study was performed in presence or absence of a specific phosphor blocking peptide (Supp. Figure S2).

Therapy and Outcome
With the aim of normalizing the day-time and night-time plasma amino acid concentration, different baseline hyperproteic diets were designed. For a range of technical reasons, only patient 1 has so far received treatment based on the findings of the present work. A diet of 2g/kg/day of protein plus a BCAA supplement of 100 mg/kg/day 4 times per day was first tried but found insufficient. The increase to a baseline natural protein intake of 3.5g/kg/day plus 100 mg/kg/day, involving continuous feeding by gastrostomy overnight until 8.00 h, with BCAA input every 5 h (the last dose at 23.00 h) provided the best results (Table 1). Six months later (when P1 was aged 5 years and 4 months), the patient showed less hyperactivity (including a longer attention span) and reduced irritability. Communication and socialization was also improved. Gross motor skills improved significantly, with the patient able to walk autonomously for a few seconds. A Vineland Adaptive Scale test (Supp. Table S2) showed objective improvements in all these areas compared to before treatment. Growth variables also improved, but remained below normal for the patient’s age.

DISCUSSION

The present paper describes two patients with psychomotor and developmental delay, microcephaly and behavioural abnormalities, who were examined for inherited metabolic disease, and found to have either a null p. R174Gfs1* or missense p.L389P homozygous BCKDK genotype. They suffered no episodes of decompensation or signs of apparent neurodegeneration, suggesting their having “static” encephalopathy. The presence of a persistent, significant reduction in body fluid BCAA levels, but normal levels of other amino acids, drew the attention of biochemical genetic laboratory scientists.

The present results clearly show that, in patient’s fibroblasts, BCKDK deficiency causes increased BCKD activity and rapid BCAA catabolism (Fig. 1 B). This results in low (P2) or even extremely low (P1) body fluid BCAA concentrations (Fig. 1 A). Experiments performed
using immortalized patient fibroblasts showed the mechanism of loss of function in both mutants to be the almost complete prevention of BCKDK expression (Fig. 4). This might be an expected consequence of the p.R174Gfs*1 premature STOP codon mutation, but is not so easily understood as a consequence of the p.L389P missense mutation. The expression of L389P BCKDK in E. coli, however, rendered a stable protein with almost no intrinsic kinase activity (Fig. 3). Purified rat BCKDK exists in an oligomeric state in equilibrium between dimer and tetramer forms (Machius et al., 2001); the protein has been crystallized in dimer form. Interestingly, Leu389 is located at the interface between dimers (Supp. Figure S3). The lack of expression and intrinsic activity of p.L389P-affected protein suggests that BCKDK dimerization is essential for its kinase activity and protein stability in vivo. Further studies of mutant L389P may reveal the biological significance of BCKDK dimerization. It cannot be ruled out that wild-type spliced protein (p.R174G) is expressed in the major BCAA-catabolising organs (i.e., liver, brain and skeletal muscle) of P1. If it is the case, the protein product would be hypomorphic, as suggested by its significant residual kinase activity in vitro (>50% of wild-type protein). However, the severity of P1’s condition suggests that the wild-type spliced protein makes only a small contribution towards his phenotype.

The first description of BCKDK-deficiency disease was that of a Mendelian form of autism with comorbid intellectual disability and epilepsy (Novarino et al., 2012). However, little information was provided on clinical signs, developmental milestones or neuropsychological evaluation scores. The present patients suffered problems of communication, social interaction, behaviour, and showed some of the classic stereotypes of autistic spectrum disorders, but the most prominent manifestation was a global and severe neurodevelopmental disorder affecting cognition and motor function. It has been described that 30 to 50% of patients with developmental delay or mental retardation have different types of associated neuropsychiatric symptoms, including autistic features (Harris et al., 2008). Therefore, the
present patients might be described as being affected by a neurodevelopmental disease that involves different signs: psychomotor delay, epileptic activity and features of autism. These signs, which are common to many disorders of impaired brain function during early development, are manifestations of abnormal synaptic communication.

BCKDK\(^{-/-}\) mice, which were described some years ago (Joshi et al., 2006), have provided a model for probing the effect of chronic BCCA deficiency on brain function and growth. Adult mice of this type develop a complex neurological condition characterized by tremors, seizures, motor defects (hind limb flexion throughout life), and retarded growth (Joshi et al., 2006). No significant growth abnormalities were reported in the first description of patients with BCKDK deficiency, although the present patients both showed growth curves below the normal percentiles. The brain was particularly affected (microcephaly). Despite the patients’ deficient isoleucine levels, especially those of P1, no acrodermatitis enteropathica-like syndrome – which is seen in extreme cases of organic acidemia and in patients with maple syrup urine disease (MSUD) following a restricted BCAA diet (Tabanlioglu et al., 2009; Scholl-Burgi et al., 2012) – was evident.

As recorded for the above BCKDK\(^{-/-}\) mice, the improvement in global brain function associated with the normalization of plasma BCAA levels (observed even in the early stages of dietary treatment in P1, the patient with the lowest BCAA levels), reinforces the idea that low brain BCAA concentrations play a major role in the pathophysiology of the described symptoms. Net nitrogen transfer from BCAA to glutamate occurs when the carbon skeleton of the BCAA is irreversibly lost via the BCKD step. A completely active BCKD might promote an imbalance in the excitatory neurotransmitter glutamate and the inhibitory neurotransmitter GABA (\(\gamma\)-aminobutyric acid) (Yudkoff et al., 1996), which might help explain both patients’ neurological phenotype.
In summary, this paper reports two new cases of BCKDK deficiency. The patient with the most severe condition in terms of type of mutation, biochemical variables and neurological symptoms, showed an improvement six months after beginning a protein-rich diet and BCAA supplementation. The present results suggest that newborns with microcephaly should have their blood amino acid concentration tested in order to detect possible BCKDK deficiency. If low levels are found, BCAA supplementation should be started to try to prevent irreversible brain damage. Since BCAAs are routinely quantified in dried blood spots by tandem mass spectrometry in expanded neonatal programs, BCKDK deficiency could be easily detected.

ACKNOWLEDGMENTS

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The authors declare no conflict of interest.

REFERENCES


Figure 1: Metabolite signature of branched-chain amino acids catabolism dysfunction.

A- Table summarizing BCAA and BCKA levels in patient blood, CSF and urine. *Controls: Reference values for 3-10 years. α-KIC: α-ketoisocaproic acid; α-KMVal: α-keto-β-methylvaleric acid; α-KIV: α-ketoisovaleric acid. B- [U-13C] α-keto-isocaproate acid was measured by HPLC/MS/MS in spent culture medium (MEM-Leu supplemented with 0.8 mM [U-13C] leucine) in which patient or control fibroblasts had been incubated for 24 h. Data normalized to number of cells and mg of protein represents the means ± SE of two replicates.

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<th>Plasma (µmol/L)</th>
<th>CSF (µmol/L)</th>
<th>Urine (mmol/mol creatinine)</th>
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<td>Patient 1</td>
<td>Patient 2</td>
<td>Control*</td>
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<tr>
<td>LEUCINE</td>
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<td>Isoleucine</td>
<td>12-17</td>
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<td>Valine</td>
<td>4-14</td>
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<td>α-KIC</td>
<td>43-85</td>
<td>70 - 151</td>
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<tr>
<td>α-KIV</td>
<td>2</td>
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Figure 2: Genetic analysis. A- Diagram of the BCKDK gene and sequence DNA surrounding the mutation (carried in homozygosis) in both patients. B- RT-PCR analysis of BCKDK mRNA in patient and control fibroblasts. DLD was used as a loading control. Sequence of BCKDK transcripts obtained from P1 and P2. C-The genomic c.520C>G change created a new 5´ donor splice site (numbers in diagram show the scores obtained using the tools at http://www.fruitfly.org/seq_tools/splice.html), provoking the skipping of a 23 bp exonic sequence. Genomic c.1166T>C is a missense change. All sequences are shown in the 5´→3´ direction.
Figure 3: Residual kinase activity of MALBP-BCKDK wild-type (WT) and mutants R174G and L389P. SDS-PAGE autoradiography of negative control (C-: MALBP), MALBP-BCKDK WT, and mutants R174G and L389P expressed in E. coli, following a kinase assay run overnight with 0.4 mM ATP $\gamma$32 (0.1mCi/mmol), 1 µg of BCKDK variants, and 2.5 µg of recombinant E1-α. A representative experiment is shown.
Figure 4: Effect of the mutations on the expression of BCKDK protein: A- Immunofluorescence microscopy showed the co-localization of Mitotracker® mitochondrial dye (red) and BCKDK (green) in BCKDK-deficient patients after infection with the lentiviral plasmid carrying wild-type BCKDK (BCKDK WT). No protein was detected upon infection with the mock vector. Nuclei were counterstained by DAPI. The scale bar represents 10 μm.

B- Representative immunoblots of mitochondrial extracts from patient and control fibroblasts infected with the same plasmid carrying the BCKDK WT, or a mock control vector (□), or un-infected, blotted against phosphor-S293 BCKD E1-alpha (1:4000) (p-BCKDE1 α), BCKDE1-alpha (1:1000) (BCKDE1 α), BCKD kinase (1:1000) and Hsp60 (1:5000, loading control).
**INFECTION**

**A**

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**p.L389P PATIENT 2**

**p.R174Gfs*1 PATIENT 1**

**B**

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</thead>
<tbody>
<tr>
<td>BCKDK WT</td>
<td>∅</td>
<td>-</td>
</tr>
<tr>
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</tbody>
</table>

- **39 kDa**
  - BCKDK
  - p-BCKDE1α

- **46.5 kDa**
  - BCKDK
  - p-BCKDE1α

- **50 kDa**
  - BCKDE1α
  - Hsp60
Table 1. Biochemical results

<table>
<thead>
<tr>
<th></th>
<th>Plasma (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patient 1 BT</td>
</tr>
<tr>
<td>Leucine</td>
<td>12-17</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4-14</td>
</tr>
<tr>
<td>Valine</td>
<td>43-85</td>
</tr>
</tbody>
</table>

BT: Before treatment. AT: After treatment. Treatment consisted of a chronic hyperproteic diet (3.5g/kg/day) plus BCAA amino acid supplementation (100 mg/kg/day every 5 h); AT values corresponds to determinations at one (*) and four months (**) after beginning treatment. Control values were established in pediatric population. +/- indicates 1 SD.