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A frame-shift deletion in the *PURA* gene associates with a new clinical finding: Hypoglycorrhachia. Is GLUT1 a new PURA target?

Lía Mayorga[a,*], Beatriz Gamboni[b], Alejandra Mampel[c], María Roqué[a]

[a] Instituto de Histología y Embriología de Mendoza (IHEM), Universidad Nacional de Cuyo, CONICET, Mendoza, Argentina
[b] Instituto de Neurología Infanto Juvenil (Neuroinfan), Mendoza, Argentina
[c] Instituto de Genética, Hospital Universitario, Universidad Nacional de Cuyo, Mendoza, Argentina

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ABSTRACT

PURA is a DNA/RNA-binding protein known to have an important role as a transcriptional and translational regulator. Mutations in the *PURA* gene have been documented to cause mainly a neurologic phenotype including hypotonia, epilepsy, development delay and respiratory alterations. We report here a patient with a frame-shift deletion in the *PURA* gene that apart from the classical PURA deficiency phenotype had marked hypoglycorrhachia, overlapping the clinical findings with a GLUT1 deficiency syndrome. *SLC2A1* (*GLUT1*) mutations were discarded, so we hypothesized that GLUT1 could be downregulated in this PURA deficient scenario. We confirmed reduced GLUT1 expression in the patient's peripheral blood cells compared to controls predicting that this could also be happening in the blood-brain barrier and in this way explain the hypoglycorrhachia. Based on PURA's known functions as a transcriptional and translational regulator, we propose GLUT1 as a new PURA target. Further in vitro and in vivo studies are needed to confirm this and to uncover the underlying molecular mechanisms.

1. Introduction

Genetic alterations in *PURA* or Pur-alpha (purine-rich element binding protein A) have been documented in humans as the origin of neurologic syndromes [1] and the cause for the 5q31.3 Microdeletion Syndrome phenotype [2,3]. PURA is expressed in brain, muscle, heart and blood and it is known to be essential for normal brain development [4,5]. Up to now, this disease has been documented to be caused by de novo, dominant mutations [1,2]. Patients with this disorder have been described with hypotonia, feeding difficulties, severe developmental delay, respiratory difficulties, pituitary dysfunction, and epileptic/non-epileptic encephalopathy associated with delayed myelination [1,6–10]. As in most neurologic diseases, these symptoms overlap with genetic and acquired encephalopathies of diverse origins, making diagnosis often a challenge.

PURA is a single-exon gene, that encodes a highly conserved multifunctional protein, member of the PUR family (Pur-alpha, Pur-beta and Pur-gamma) [11]. It is a DNA and RNA binding protein that plays an important role in cell proliferation, transcriptional regulation and mRNA trafficking [12,11]. As a member of the PUR family, it has three conserved sequence-specific repeats: PUR domains I, II and III that are responsible for the protein's main functions [11]. Each PUR amino acid repeat consists of a β-sheet domain and an α-helical domain arranged in a "whirly fold," structure in which the convex β-sheets form a surface for interaction with nucleic acids and the remaining helix portions are involved in protein-protein interactions [12,13]. PUR I and II motifs are proposed to form intramolecular peptide-peptide bonds between each other, while the PUR III domain is responsible for homo-heterodimerization with another PUR protein or interaction with other proteins [14,13] (Fig. 1). It has been shown that specifically PURA can regulate gene expression by binding directly to DNA promoters, or to different mRNAs and non-coding RNAs. It can also form DNA-mRNA-protein complexes, all features that make it an important transcriptional and translational regulator [15,16].

SLC2A1 gene (clinically better known as *GLUT1*) is a member of the GLUT family of facilitative glucose transporters, which includes 13 genes. *SLC2A1* was the first cloned and sequenced gene of the group and it encodes for GLUT1 protein [17]. GLUT1 is ubiquitously expressed in most tissues, but selectively higher in erythrocytes, brain microvessels and astroglia. Glucose is the essential substrate for brain metabolism and its transport across the blood-brain barrier depends on GLUT1 [18]. It is also important for glucose uptake in red blood cells since these cells´ metabolism is strictly glycolytic [19,20]. GLUT1 deficiency syndrome classically presents with infantile seizures (often

* Corresponding author at: IHEM, Centro Universitario UNCuyo, Libertador 80, Mendoza 5500, Argentina.
E-mail address: liamayorga@fcm.uncu.edu.ar (L. Mayorga).

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resistant to antiepileptic drugs), developmental delay, acquired microcephaly, hypotonia, spasticity, dystonia and in some cases hemolytic anemia all associated to its key biomarker: hypoglycorrhachia [21].

We report here a patient with a one-base frame-shift deletion in the *PURA* gene that presented an unusual phenotype including persistent hypoglycorrhachia. We propose a possible link between hypoglycorrhachia and PURA dysfunction postulating PURA as a GLUT1 regulator. We therefore hypothesize that mutations in *PURA* can decrease GLUT1 expression and in consequence provoke a GLUT1 deficiency-like phenotype.

2. Materials and methods

2.1. Patient consent

Written informed consent was obtained from the patient's parents who agreed to use the patient's samples for research and data for publication. The blood samples used for research purposes were obtained within diagnostic or clinical control procedures. Blood protein from 4 anonymous healthy controls were used to compare with the patient.

2.2. Genetic testings

DNA extraction: IHEM, UNCuyo, CONICET - Mendoza, Argentina.

Leukocyte DNA from the patient and both parents was extracted using CTAB/chloroform-isoamyl alcohol protocol. Briefly, blood was frozen at − 20 °C before DNA extraction. Once thawed, white blood cells were isolated following three to four Tris-EDTA ($T_{10}E_{10}$) buffer washes, which were then suspended in Cetyl Trimethylammonium Bromide (CTAB) solution (2 g/l CTAB Sigma Aldrich®, 100 mM Tris/HCl, 20 mM EDTA and 2% 2-mercaptoethanol) and incubated at 60 °C during 1 h for membrane lysis. Afterwards, chloroform isoamyl alcohol solution (24:1) was added and the sample was centrifuged. The aqueous phase was collected into a new tube and mixed with 3 volumes of ice-cold 100% ethanol. Precipitated DNA was dissolved in $T_{10}E_{0.1}$ buffer and stored at − 20 °C for further use.

SLC2A1 gene sequencing + copy number variation analysis (Leukocyte DNA): MNG laboratories® - Atlanta, GA, USA.

White blood cell DNA was studied with Sanger-sequencing (including analysis of the coding exons and the flanking donor/acceptor sequences) and Multiplex ligation-dependent probe amplification (MLPA, MRC-Holland® P138 commercial kit that includes probes on all 10 exons of the *SLC2A1* gene). With Sanger sequencing, mutations and single/few base pair deletions/duplications were studied. MLPA was used to detect larger copy number variations (≥ 1 exon).

Whole exome sequencing of the proband (Leukocyte DNA): Baylor College of Medicine® (BCM) - Houston, TX, USA; Test code 1500. White blood cell DNA from proband and both parents were sent to BCM. Complete clinical information was provided in order to focus the report. The proband's DNA was fragmented and ligated to the Illumina multiplexing paired-end adapters, followed by PCR amplification using primers with sequencing barcodes (indexes). For target enrichment/exome capture procedure, the pre-capture library was enriched by hybridizing to biotin-labeled VCRome 2.1 in-solution exome; additional probes for over 3600 Mendelian diseases were included in the capture in order to improve the coverage. For massive parallel sequencing, the post-capture library DNA was subjected to sequence analysis on Illumina HiSeq platform for 100 base paired-end reads. Quality control of the sequencing data: > 70% of reads aligned to target, > 95% target base covered at > 20X, > 85% target base covered at 40X, mean coverage of target bases > 100X. SNP concordance to genotype array > 99%. The output data from Illumina HiSeq was mapped to the reference haploid human genome sequence GRCh37/hg19. Variant calls were performed using Atlas-SNP and Atlas-indel in house developed by BCM Human Genome Sequencing Center®. Variants related to patient's phenotype were Sanger sequenced in proband and parents.

PURA Sanger sequencing on leukocyte DNA from the proband and both parents: Baylor College of Medicine® - Houston, TX, USA. Sanger sequencing of the altered region in the patient found by WES was studied in the patient and both parents.

In silico protein function prediction: IHEM, UNCuyo, CONICET - Mendoza, Argentina.

The online tool *predictprotein* (www.predictprotein.org) was used to schematize the patient's specific defect and predict the protein's function, localization (LocTree2), secondary structure (PROFphd) and binding sites (InteractionSites).

GLUT1 expression assays: IHEM, UNCuyo, CONICET - Mendoza, Argentina.

GLUT1 Western Blot assays on red blood cell (RBC) membranes and white blood cells (WBC).

WBC protein extraction: Upon withdrawal, 3 ml of blood was frozen at − 20 °C until being processed. Once thawed, WBC were isolated with three to four $T_{10}E_{10}$ buffer washes. The white cell pellet was immersed in 500 µl Trizol® reagent (Thermofisher scientific®) and homogenized in a ultra turrax homogenizer. Protein was obtained according to Trizol® protocol and diluted in 1% Sodium dodecyl sulfate (SDS).

RBC protein extraction: Parting from the supernatant from the first $T_{10}E_{10}$ wash used to separate leukocytes (see above), this was centrifuged at 4 °C: first 5 min at 1800 rpm and then the supernatant was centrifuged 45 min at 13500 rpm. The pellet was suspended in Triton lysis buffer (Triton 0.5%, NaCl 150 mM, EDTA 5 mM, Tris-HCl 1 M). Protein concentration was measured using spectrophotometry (LABOCON®).

Western Blot assays: Equal amounts of solubilized protein (30 µg for WBC and 40 µg for RBC) obtained from the patient and 4 healthy controls were run on a 12.5% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. The membrane was blocked in 5% low fat milk-PBS solution and then incubated overnight at 4 °C with primary antibodies: Anti-GLUT1 1:500 (rabbit- Alomone Labs® Cat #: AGT-021) and Anti- β-actin 1:1000 (mouse- GenScript® Cat# A00702) followed by PBS-Tween washes and then secondary antibody incubation (2 h at room temperature): Horseradish peroxidase secondary antibodies: mouse (Jackson ImmunoResearch Labs Cat# 223–005-024) and rabbit (Jackson ImmunoResearch Labs Cat# 323-005-021) 1:50,000. Bands were developed using chemiluminescence (ECL Merck-Milipore reagent®) and visualized with a LAS Fujifilm 4000 system (GE Healthcare Life Sciences®). Protein bands were quantified using Image J v. 1.5. GLUT1 was relativized to beta actin for quantification, and then controls were relativized to patient's value for statistical analysis which was performed using GraphPad Prism v.5®, (One-sample Student's *t*-test).

3. Results

3.1. Patient's clinical findings

Female, second child of a non-consanguineous couple. Born at term with normal weight from an uneventful pregnancy, discharged on her 2nd day of life without complications. She was admitted to hospital on day 7 due to failure to thrive, poor suction and dehydration. She was noted to be profoundly hypotonic and continued admitted for further studies. Parents mentioned abnormally frequent hiccupping during the first week of life. Seizures and apneas started on day 8, needing ventilator support for 3 days. First brain MRI + spectroscopy (in the neonatal period) showed cerebellar vermis hypoplasia, cystic dilatation of the quadrigeminal cistern and megacysterna magna with normal spectroscopy. EEG in this period had a burst suppression pattern. Central and peripheric apneas were detected needing Oxygen supplementation until now. Her first cardiologic evaluation including ECG and echocardiogram was normal. In the neonatal period she had anemia that required several red blood cell transfusions with normal

hematological work up. Karyotype was normal: 46,XX. Extensive metabolic studies including organic acids, plasmatic and cerebrospinal fluid (CSF) amino acids, acylcarnitines, biotinidase activity, urine sulfites, transferrin isoelectric focusing, neurotransmitter levels in CSF and very long chain fatty acids were all normal or inconclusive. The only abnormal metabolic finding was hypoglycorrhachia in three independent lumbar punctures (LPs) performed in the first 3 months of life: 19, 36 and 33 mg/dl (normal values > 40 mg/dl). The CSF to blood glucose ratios were also low: 0.25, 0.55 and 0.39 (normal value > 0.55). Hypoglycemia was never detected. CSF lactic acid was measured twice and found in the low range: 0.44 and 0.5 mM (normal values: 1- 2 mM). It is important to mention that the child was free of seizures at the time of the LPs. With this clinical setting and biochemical picture (hypoglycorrhachia and low CSF lactic acid) GLUT1 deficiency was suspected [21,22] and therefore a ketogenic diet was installed at 3 months of age. Seizures were well controlled during the first year with Vigabatrin at a low dose. However, infantile spasms with a hypsarrhythmic EEG started when she turned one, within the ketogenic diet treatment, requiring Vigabatrin + Valproic acid to control them. A second MRI at 14 months showed bihemispheric hypomyelination. The ketogenic diet was maintained for 10 months until molecular diagnosis was completed. KetoCal® formula was used (Fat : Carbohydrate + Protein proportion = 4:1) along with carnitine and vitamin supplementation. There was no objective response to it: as mentioned above, seizures became difficult to control within the diet, and when the diet was withdrawn the clinical setting did not change for better nor for worse.

Despite her profound hypotonia and poor head control she has been able to be orally fed with normal weight/height gain and normal head growth until now (24 months). She has acquired good social milestones (smiles, connects with people and waves her hand), although still cannot sit on her own and is non-verbal.

During the first months of life she developed a left ventricular hypertrophic myocardiopathy, predominantly due to septum enlargement. Her heart has always maintained normal function, not requiring medication at the time. It is important to mention that the myocardiopathy was detected before starting the ketogenic diet, ruling out Selenium deficiency as the cause [23].

She developed intermittent predominantly left strabismus. Visual evoked potentials were bilaterally abnormal and electroretinography was normal. Table 1 shows the patient's clinical findings comparing new and previously reported PURA and 5q31.3 microdeletion syndrome symptoms.

3.2. Genetic diagnosis and protein functionality prediction

SLC2A1 sequencing + copy number variation analysis: With the suspicion of GLUT1 deficiency, the *SLC2A1* gene was studied by Sanger sequencing and MLPA on patient's white blood cell DNA. Mutations and copy number variations were discarded. GLUT1 deficiency has been reported mainly due to mutations or single to few base pair deletions/duplications in the *SLC2A1* gene (85–89%) and in less frequency produced by exon or multi-exon deletions of the gene (11–14%) [19,24].

With this negative result, Whole Exome Sequencing was pursued.

Whole exome sequencing (WES) on proband's leukocyte DNA: An unreported de novo heterozygous deletion was found at the nucleotide position 586 (c.586delA) of the *PURA* gene, provoking a frame-shift of the reading frame at the amino acid 196 (p.I196fs). The parents *PURA* gene was Sanger sequenced in this region, revealing no mutation. The predicted outcome of this frame-shift is a truncated dysfunctional protein with 223 amino acids (instead of the 322 corresponding to the wild type form). This truncated protein is predicted to maintain its secondary structure (loop > β-strand > α-helix) and nuclear localization. However, it loses the PUR III domain probably limiting PURA homo-heterodimerization and interaction with other proteins (Fig. 1). The transcriptional/translational role of the altered PURA protein undoubtedly must be comprised. This pathogenic variant of the protein is proposed to explain her clinical phenotype.

In line with the *SLC2A1* Sanger sequencing results, no variants were reported on the *SLC2A1* gene in the WES analysis and it had a 100% coverage at 20X.

Additionally, on the WES analysis, two missense variants of unknown significance (VUS) were reported in the *SETD2* gene: c.509C > T (p.A170V) and c.1775C > A (p.T592K). They were both confirmed by Sanger sequencing and found inherited in cis from the mother. The first mutation by SIFT [25] and PolyPhen-2 [26] analysis was classified as tolerable/benign and the latter as damaging/benign. Mutations in the *SETD2* gene cause Luscan-Lumish syndrome (MIM 616831) which includes postnatal overgrowth, macrocephaly, obesity, speech delay, and advanced carpal ossification as clinical features. Hypoglycorrhachia has not been associated to this disease. Since the clinical features of this disorder did not overlap with the patient and the mother was totally asymptomatic, these VUS were underestimated as the cause of the patient's disease.

3.3. GLUT1 expression in patient's blood cells

Even though mutations in *SLC2A1* gene had been discarded, the marked hypoglycorrhachia made us ask whether its expression could be altered in the patient. By Western Blot assays we could confirm a significantly reduced expression of GLUT1 in the patient's red blood cells ($p = 0.04$, One- tailed one sample Student's *t*-test) and white blood cells ($p = 0.06$, One- tailed one sample Student's *t*-test) (Fig. 2).

4. Discussion

We have presented a patient with a monoallelic defect in the *PURA* gene that causes the expression of a truncated dysfunctional protein, explaining the phenotype. This patient's clinical setting with marked hypoglycorrhachia and hypertrophic myocardiopathy expands PURA deficiency's phenotype since this has not been previously reported in patients [1,2,6] nor in PURA deficient mice models [4,5]. We did not deepen our research in the cardiomyopathy, but PURA as a transcription regulator of genes such as alpha actin [27] and alpha myosin [16] in cardiomyocytes, could be responsible to cause cardiac abnormalities when dysfunctional.

We did extend our observations regarding the hypoglycorrhachia and were able to prove a GLUT1 downregulation in the patient's peripheral blood cells. It is reasonable to infer that this decrease of GLUT1 could also be happening in the blood-brain barrier and in this manner explain the detected hypoglycorrhachia. Since mutations in the *SLC2A1* gene were discarded, and based on the fact that PURA is known to be a transcriptional/translational regulator, we propose that PURA could be

Table 1
Patient's clinical features. Patient's clinical features comparing previously reported (left column) and new findings (right column) in PURA and 5q31.3 microdeletion syndrome. Highlighted with (*) and bold letters are GLUT1 deficiency overlapping symptoms.

Previously reported clinical features in PURA and 5q31.3 microdeletion syndrome present in the patient	New clinical features in this patient
Hypotonia, development delay	**Hypoglycorrhachia***
Apneas, oxygen requirement	**Low lactic acid in CSF***
Epilepsy	**Anemia** requiring transfusions in the neonatal period*
Strabismus/abnormal visual evoked potentials	
Cerebellar hypoplasia, hypomyelination	Hypertrophic myocardiopathy
Persistent hiccupping in the neonatal period	EEG with a burst-suppression pattern in the neonatal period

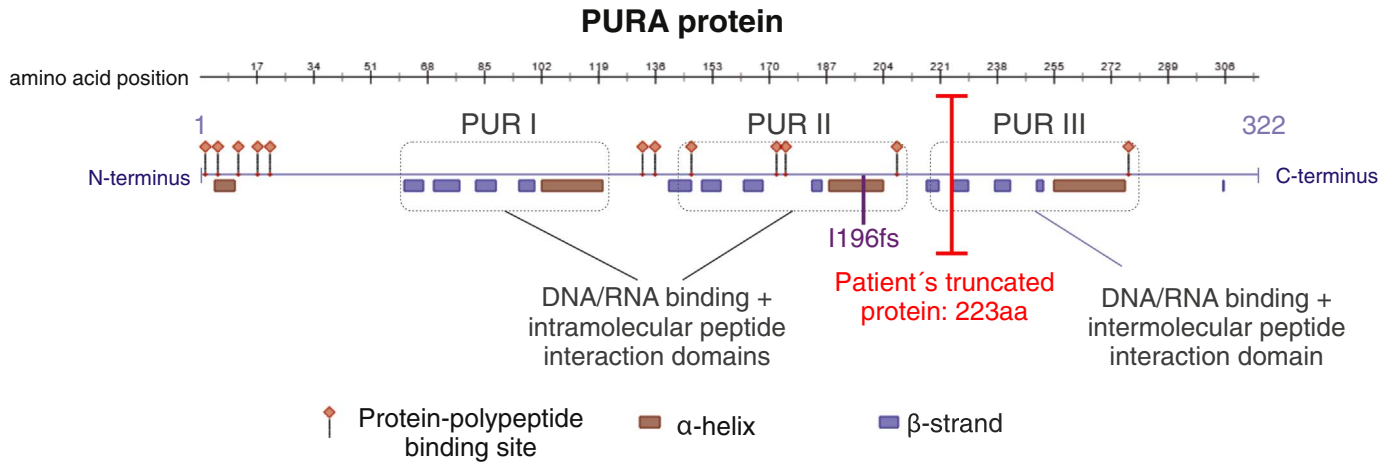

Fig. 1. PURA protein structure: wild-type vs. patient's truncated protein.
The 322 amino acid PURA wild-type protein structure was obtained through the online tool: www.predictprotein.org. The three PUR domains are schematized based on literature [11,13]. Amino acid position is indicated on top. Patient's frame-shift mutation is marked with a bar at amino acid I196, generating a premature stop codon 27 amino acids downstream (indicated with a larger bar at position 223). The truncated protein is expected to lose the PUR III motif. (figure modified from www.predictprotein.org).

a facilitator of the expression of GLUT1. Therefore, mutations affecting its function would reduce the amount of GLUT1. These findings propose an expansion of the phenotype associated to PURA mutations and presents GLUT1 as a yet unknown PURA target.

PURA binds to purine rich single and double-stranded nucleic acids in a sequence specific manner. (GGN)n [28], (CGG)n [14], (CAG)n [29], (G_4C_2)n [30,31] repeats and the CCCGGCC [32] sequence are all known PURA binding sites [30]. It binds to Guanines and additionally contacts with the phosphodiester backbone. Apart from its ability to bind RNA and ssDNA, PURA has a dsDNA-destabilizing activity in an ATP-independent fashion [33,34], important for DNA replication and transcription regulation. As a transcription activator, PURA contacts the purine-rich strand of promoter regions and displaces the pyrimidine-rich strand, allowing the binding of other proteins, transcription factors and therefore transcription activation [34]. PURA also has a role in protein translation binding to non-coding RNAs and mRNAs, as in compartmentalized translation in neurons [15]. Based on PURA's known functions, GLUT1´s regulation could be associated to PURA-dependent transcription activation or translation regulation. The specific PURA-binding sequences mentioned above are all present in the *SLC2A1* promoter regions and/or the *SLC2A1* mRNA, making GLUT1 a possible PURA target.

We consider that hypoglycorrhachia could be added to the PURA syndrome clinical spectrum, and so *PURA* should be tested in a hypoglycorrhachia setting after GLUT1 mutations are ruled out. The ketogenic diet, known treatment for GLUT1 deficiency [35], did not show any benefit in our patient. This could be due to the many possible *PURA* targets that are dysregulated in PURA deficiency, making GLUT1 not the only one. Anyhow, ketogenic diet might be useful in some PURA patients when seizures don't respond to antiepileptic drugs, as in other refractory epilepsy settings [36].

To conclude, we propose GLUT1 as a new PURA target. Further studies in other PURA patients, PURA deficient mice and PURA/GLUT1 manipulation experiments in cell culture are needed to confirm this statement and to unravel the possible molecular mechanisms.

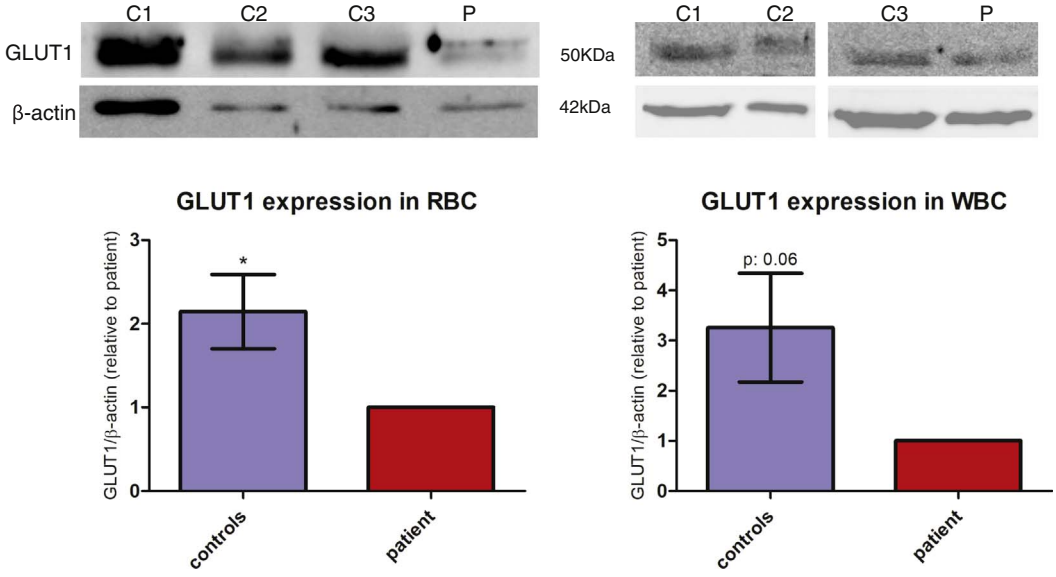

Fig. 2. GLUT1 expression in blood cells.
GLUT1 Western Blot assays in red (RBC) and white blood cells (WBC). C1, C2, C3: controls. P: patient. Patient's GLUT1 expression is reduced comparing with controls in RBC ($p = 0.04$) and in WBC ($p = 0.06$). GLUT1 shows two bands that have been described as different glycosylated forms [37]. β-actin was used as a housekeeping protein. Quantification was performed as GLUT1/β-actin and relativized to patient's value. One-tailed one sample Student's *t*-test was used for statistical analysis.

Conflict of interest

The authors declare that they have no conflict of interest nor competing financial interests.

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