

SCREENING SLC2A1 GENE FOR SEQUENCE AND COPY NUMBER VARIATIONS ASSOCIATED WITH GLUT-1 DEFICIENCY SYNDROME

GLUT-1 EKSİKLİĞİ SENDROMU İLE İLİŞKİLİ SLC2A1 GENİNDE YER ALAN DİZİ VE KOPYA SAYISI VARYASYONLARININ İNCELENMESİ

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Cite this article as: Ornek Erguzeloglu C, Ozdemir O, Kesim Y, Karacan I, Kara B, Bebek N et al. Screening SLC2A1 gene for sequence and copy number variations associated with GLUT-1 Deficiency Syndrome. J Ist Faculty Med. Published online October 21, 2019. doi: 10.26650/IUITFD.2019.0064

ABSTRACT

Objective: Glucose transporter-1 deficiency syndrome (GLUT1-DS) is defined as a metabolic encephalopathy that is associated with heterozygous and usually *de novo* pathogenic variations in the *SLC2A1* (solute carrier family2 member1) gene.

Materials and Methods: In this study, all coding exons and neighboring intronic regions of *SLC2A1* were Sanger sequenced in 12 patients with clinically suspected GLUT1-DS. For *de novo* variations revealed after sequencing and segregation analysis, we also performed genome wide Single Nucleotide Polymorphism (SNP) genotyping to confirm parental relatedness with the proband. In patients without any sequence variations, real-time quantitative real-time polymerase chain reaction (qPCR) was applied to determine the presence of any copy number variations (CNV).

Results: Sanger sequencing followed by bioinformatics analysis, segregation in the family and SNP array genotyping revealed two novel and *de novo* pathogenic variations associated with the GLUT1-DS phenotype in 2 patients. qPCR results were com-

ÖZET

Amaç: GLUT1 eksikliği sendromu (GLUT-1ES) bebeklik çağında başlayan metabolik bir ensefalopati olarak tanımlanmıştır. Kolaylaştırılmış glikoz taşıyıcısı olan GLUT1'i kodlayan *SLC2A1* genindeki *de novo* patojenik varyasyonlardan kaynaklanır.

Gereç ve Yöntem: Bu çalışma kapsamında, GLUT1-ES klinik şüphesi olan 12 hastada *SLC2A1* genin tüm ekzonları Sanger dizileme method ile taranmıştır. *De novo* varyantlarının anne baba çocuk üçlüsü açısından uyumluluğu Tek Nükleotid Polimorfizmi (SNP) genotiplemesi ile yapılmıştır. Sanger analizinde herhangi bir değişikliği olmayan hastalarda, gerçek zamanlı kantitatif PZR (Polimeraz Zincir Reaksiyonu) analizi ile kopya sayısı değişimleri incelenmiştir.

Bulgular: Sanger dizileme, biyoinformatik analiz, aile segregasyonu ve SNP genotipleme yaklaşımlarının ardarda uygulanması ile 2 hastada GLUT1-ES fenotipiyle ilişkili iki yeni ve *de novo* patojenik varyasyon tespit edilmiştir. Gerçek zamanlı qPZR sonuçları ise bir başka hastada *SLC2A1* geninin bir kopya kaybıyla uyum-

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Başvuru/Submitted: 19.08.2019 • **Revizyon Talebi/Revision Requested:** 18.09.2019 •

Son Revizyon/Last Revision Received: 04.10.2019 • **Kabul/Accepted:** 10.10.2019 • **Online Yayın/Published Online:** 21.10.2019

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patible with one copy loss of *SLC2A1* gene in another patient. All variations identified herein are likely to have caused null alleles and resulted in GLUT1-DS through haplo insufficiency.

Discussion: In this study we used a series of molecular genetic approaches in order to identify all possible variations in *SLC2A1* that may be associated with GLUT1-DS. This collective effort facilitated diagnosis in 3 patients.

Keywords: Glucose transporter-1 deficiency syndrome (GLUT1-DS), *SLC2A1*, *de novo* variations, CNV analysis, SNP array

lu bulunmuştur. Tespit edilen 3 varyasyonun da *SLC2A1* geninin bir allelin fonksiyonunu tamamen ortadan kaldırarak haplo yetersizlik mekanizması ile hastalığa yol açtığı öngörülmüştür.

Tartışma: Bu çalışma ile pek çok farklı moleküler genetik teknik ve analizler kullanılarak GLUT1-ES hastalığında gen seviyesindeki olası tüm değişikliklerin belirlenmesi hedeflenmiş; klinik tanıya katkı sağlanmıştır.

Anahtar Kelimeler: GLUT1 Eksikliği Sendromu, *SLC2A1*, *de novo* varyasyon, CNV analizi, SNP dizileme

INTRODUCTION

GLUT1 deficiency syndrome (GLUT1-DS) is caused by heterozygous pathogenic variations of the *SLC2A1* gene. *SLC2A1* encodes GLUT1 protein, which acts as the primary glucose transporter along the blood-brain barrier. *SLC2A1* gene is located on chromosome 1p34.2 and its largest transcript consists of 10 coding exons (1).

Lack or absence of GLUT1 protein leads to insufficient glucose levels for cerebral metabolism (1). Classical GLUT1 encephalopathy is characterized by infantile-onset seizures, developmental disability, complex motor disorders, and variable microcephaly. Although *SLC2A1* variations are known to cause GLUT1-DS, there is still need for determination of proper phenotype-genotype correlations. Most patients carry heterozygous *de novo* mutations in the *SLC2A1* gene. In familial cases, autosomal dominant inheritance is observed with full penetrance and autosomal recessive transition has rarely been reported (2). In clinical practice, the clinically available

distinctive biomarker for GLUT1-DS is a low concentration of glucose in cerebrospinal fluid (CSF) (<50 mg/dl or CSF-to-blood glucose ratio <0.45). Early diagnosis is critical for an effective etiological therapy (3,4).

In this study we set out to identify possible pathogenic variations associated with GLUT1-DS in the *SLC2A1* gene in 12 patients. Various molecular techniques including Sanger sequencing, SNP array analysis and real-time quantitative PCR (qPCR) were used throughout this process. The scientific importance of this study is to contribute to the discussion of diagnosis genetic causes of rare neurological diseases like GLUT1 deficiency syndrome and to determine the possible phenotype-genotype correlations.

MATERIALS AND METHODS

A cohort of 12 individuals with clinically suspected GLUT1-DS were recruited to the study by the Pediatric Neurology department at Kocaeli University's Medical Faculty (Table 1). DNA was isolated from peripheral

Table 1: Review of clinical findings in the study cohort.

Patient Number	Sex	Age at diagnosis	CSF glucose concentration	Parental Cons.	Clinical Phenotype
E11-169	F	9 y	31mg/ml	Yes	microcephaly, dyskinetic movement disorder, ataxia, mental retardation, epilepsy
E13-83	F	17 y	35 mg/ml	No	Dyskinetic movement disorder, mental retardation
E15-16	F	1 y	48 mg/ml	No	mental retardation and early onset childhood absence epilepsy
E15-19	F	1 y	28 mg/ml	No	acquired microcephaly, dystonia, epilepsy, psycho-motor retardation
E15-30	F	5 y	55mg/ml	No	Early onset childhood absence epilepsy
E15-34	F	10 y	35 mg/ml	No	Early onset childhood absence epilepsy
E15-40	F	5 y	32 mg/ml	No	Early onset childhood absence epilepsy
E15-47	F	7 y	53 mg/ml	No	Early onset childhood absence epilepsy
E16-16	M	6 y	< 20mg/ml	No	Early onset childhood absence epilepsy
E16-24	F	10 y	< 20mg/ml	No	Early onset childhood absence epilepsy
E17-20	F	13 y	< 20mg/ml	No	Early onset childhood absence epilepsy
E17-79	F	8.5 y	52 mg/ml	No	Early onset childhood absence epilepsy

CSF: cerebrospinal fluid; Cons.: Consanguinity; F: Female; M:Male; Y: year

blood of all patients and their family members whenever required. The DNA was first analyzed for sequence variations via Sanger sequencing and then subjected to trio SNP array genotyping only for trios with *de novo* variants. Real time qPCR method was additionally used for detecting any possible copy number changes that would be missed by Sanger sequencing. The study protocol was approved by Istanbul University, Istanbul Faculty of Medicine, Clinical Ethics Committee (2017/493; 12/05/2017).

Genomic DNA Isolation

Peripheral blood samples were obtained in EDTA tubes and DNA was isolated using Qiagene maxi and/or mini kits as suggested. Also, 2.5 ml of peripheral blood samples of patient E11-169 and her parents were collected in PAXgene Blood RNA Tubes and RNA was isolated using PAXgene Blood RNA Kit according to the manufacturer's instructions.

Screening *SLC2A1* for sequence variations

All coding exons and neighbouring intronic regions in *SLC2A1* gene had been amplified using 10 primer pairs designed for this study (Table 2). A touch down PCR (TD-PCR) protocol was utilized for this purpose, which can increase specificity, sensitivity and yield, without the need

for struggle optimizations and/or the redesigning of primers (5,6). Each amplicon was Sanger sequenced and analyzed for sequence variations using CLC Workbench (8). Variants identified were then annotated with ENSEMBL Variant Effector Predictor Tool (VEP) for detecting the consequence of these variants on current transcript versions, retrieving up-to-date population frequency data and predicting the possible impact of amino acid substitutions on the structure and function of GLUT1 protein via PolyPhen and SIFT (<https://www.ensembl.org/info/docs/tools/vep/index.html>). Splicing consequence of splice region variants were analyzed through 'Human Splicing Finder (HSF)' (<http://www.umd.be/HSF/>). For variations with potential pathogenic effect, familial segregation analysis was performed. All variations were annotated using the only consensus codes database transcript of *SLC2A1*, namely ENST00000426263.8 (NM_006516.3) as a reference.

Parental confirmation by SNP Array

According to the American College of Medical Genetics (ACMG) Standards Guidelines published in 2015 (8), it is necessary to show parental relatedness with the proband for the confirmation of *de novo* variants. We performed SNP Array genotyping using Illumina Human Cyto SNP-12

Table 2: Primer sequences to amplify 10 *SLC2A1* fragments and exons covered by these fragments. Some amplicons were designed as overlapping fragments.

Fragment	Exons involved	Fragment Length	Fragment GC content	Primer Sequences
Fr1	ex 1	484bp	74%	GCCGGGGTCCTATAAACGCTAC CGCTAGATCCGAAGCCCATC
Fr2	ex 2	535bp	54%	AATGAGACCCCCAAGAATCC CCAGAAAACCTGGCTGGAGAG
Fr3	ex 3-4	558bp	60%	GGAAAAGGAAGACTGGGTCC ACATACATGGGCACGAAGC
Fr4	ex 4b	535bp	65%	ACTGGGCAAGTCCTTTGAG GTGTCCAGCACAGAGAATGG
Fr5	ex 5-6	466bp	71%	CTGAAGCAGCAGCTGACACAAAG GGAACAGCTCCAGGATGGTGAC
Fr6	ex 6-7	561bp	58%	TGAGCCACCCTCACCTTCC TGGCATAACAGGCTGCTGC
Fr7	ex 7-8	609bp	68%	TTCCCTTTAGACCCCAAGC TATGAAGCCCAGGCAAACCTC
Fr8	ex 8	442bp	70%	CTCTGCCCACAGCTGTTTGTG GGGGTTGCTGTGAAGATGAATTG
Fr9	ex 9	359bp	54%	GGATCCATCACAAACCCAGTC TTCCTCCTCAGCATGATTCC
Fr10	ex 10	415bp	56%	TCCAAAGTCCTACAGCCAGG CTTCTGGACATCATTGCTGG

Bead Chip kit in two parent offspring trios. The data was exported from Genome Studio platform and analyzed using an in-house approach, in which only 'informative SNPs (iSNP)' were selected for each trio. For autosomal SNPs, iSNPs are described as SNPs that are alternatively homozygous in each parent. For iSNPs the child is an obligate heterozygous. As an example if we choose all SNPs with 'AA' genotype in the father and 'BB' genotype in the mother, the offspring should essentially be 'AB'. A significant degree of heterozygosity for the offspring in the trio for iSNPs will assess biological relatedness (9).

Real-Time Quantitative Polymerase Chain Reaction (qPCR) Approach

In order to detect heterozygous copy number variations that may be missed by Sanger sequencing approach, we utilized real time qPCR approach using Roche Light Cyclers 480 with SYBR Green I Master mix in 7 patients for over 9 *SLC2A1* fragments. Herein, we aimed to map CNVs at the level of exonic resolution. Delta-delta Ct approach was used for data analysis using *PCBP3* (Poly RC Binding Protein 3) gene as a reference. For this approach, primer pairs presented on Table 2 were used except for fragments 1, 6 and 7. For these regions new primer pairs were designed. Nevertheless, fragment 1 could not be optimized for qPCR probably due to a relatively high GC content. Therefore, qPCR was performed for 9 fragments. New primer pairs for fragments 6 and 7 are as follows: Fr6F:ATCAACCGCAACGAGGAG, Fr6R:ACCATGCACTTGACCAGA, Fr7F:CCCACATCCACTGCTACAGA and Fr7R:TAGTGCCCTTCTGAACCCAC.

Gene Expression Level Analysis

Patient E11-169 and her parents were tested using mRNA expression analysis of the *SLC2A1* gene to investigate any effects on the mRNA levels. For each subject, 1 µg total RNA was reverse transcribed using First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay was performed triplicate using Roche Light Cyclers 480 with SYBR Green I Master mix and primers located on different exons (forward primer for Fr4 and reverse primer for Fr6 as indicated in Table 2). Delta-delta Ct approach was used for data analysis using *ABL* gene as a reference. Statistical significance was tested using one-way ANOVA.

***Reasons for choosing *ABL* and *PCBP3* as reference genes; stability, no change in the tissues or cells under investigation, these genes have the closest base length to the target gene. The reason for including the *ABL* gene as a second reference was that it is more compatible and optimized for the mRNA sequence step than *PCBP3* gene.

RESULTS

Interpretation of Sequence Variants in *SLC2A1*

8 different single nucleotide variations were identified in 7 patients with a suspected phenotype of GLUT1-DS. Two of them (NM_006516.3: c.680-1G>C and NM_006516.3: c.542delG) were assessed to be associated with the GLUT-1 deficiency syndrome as they were found to be novel and *de novo* (Figure 1; Table 3). Trio SNP analysis was performed for families of E11-169 and E13-83 as the

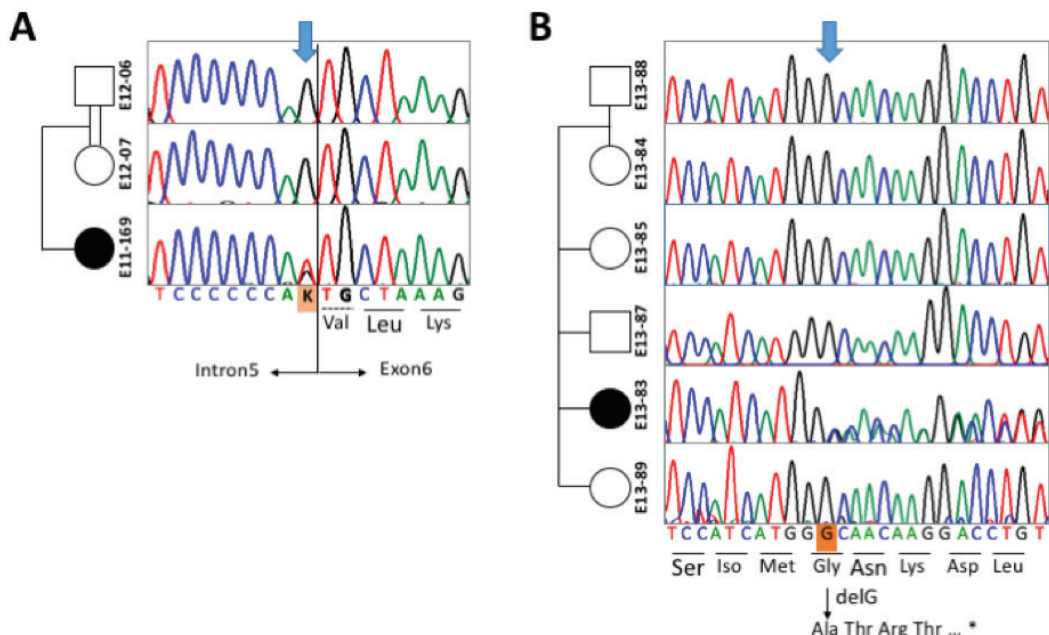


Figure 1: Segregation analysis of (a) NM_006516.3: c.680-1G>T and (b) NM_006516.3:c.542delG in the relevant families indicate a *de novo* pattern for the variations. K: ambiguity code for G or T.

Table 3: *SLC2A1* variants identified through Sanger sequencing

Patient ID	Variant ID	Seg.	c.DNA	Protein	MAF	Variant Type	Exon	Intron
E11-169	Novel	<i>de novo</i>	c.680-1G>T	-	-	splice_acceptor	-	5/9
E13-83	Novel	<i>de novo</i>	c.542delG	p.(Gly181Alafs*10)	-	frameshift	5/10	
E17-79	rs11537640	NP	c.-197A>C	-	0.23 (G)	5_prime_UTR	1/10	
E15-40, E17-20, E17-79	rs1385129	NP	c.45C>T	p.Ala15=	0.24 (A)	synonymous	2/10	
E17-79	rs76672402	NP	c.312C>T	p.Phe104=	<0.01 (A)	synonymous	4/10	
E17-79	rs755559487	NP	c.384C>T	p.Ile128=	<0.01 (A)	synonymous	4/10	
E15-30, E15-47, E17-20, E17-79	rs11537641	NP	c.399C>T	p.Cys133=	0.14 (A)	synonymous	4/10	
E15-30, E15-47, E17-20, E17-79	rs2229682	NP	c.588G>A	p.Pro196=	0.12 (T)	synonymous	5/10	

NP: not performed; Seg: Segregation

de novo status of the variants gives strong support for the pathogenic status if both maternity and paternity can be confirmed (8). Accordingly, iSNP analysis in both the trios of E11-169 and E13-83 resulted in 99.9% correlation for 11,385 and 16,015 iSNPs, respectively.

The *de novo* variant identified in E11-169 (NM_006516.3: c.680-1G>T; hg38: chr1:42,929,781C>A) is a novel splice region variant. At the same locus, another splice variant (CS057229; NM_006516.3: c.680-1G>C; hg38: chr1:42,929,781C>G) previously associated with GLUT1-DS, can be interpreted as new evidence in favor of the

novel variant identified herein (10). HSF analysis for both alterations (NM_006516.3: c.680-1G>T;C) revealed alteration of the wild type acceptor site, so it is possible that both variants are affecting splicing. The other novel and *de novo* variant in E13-83 is a single base deletion resulting in a frameshifting effect on translation (NM_006516.3:c.542delG; p.(Gly181Alafs*10); hg38:chr1:42,930,010delC). For both variations, protein truncation can be speculated, but it is likely that both alleles have a null effect possibly due to translation dependent nonsense-mediated decay. This effect well cor-

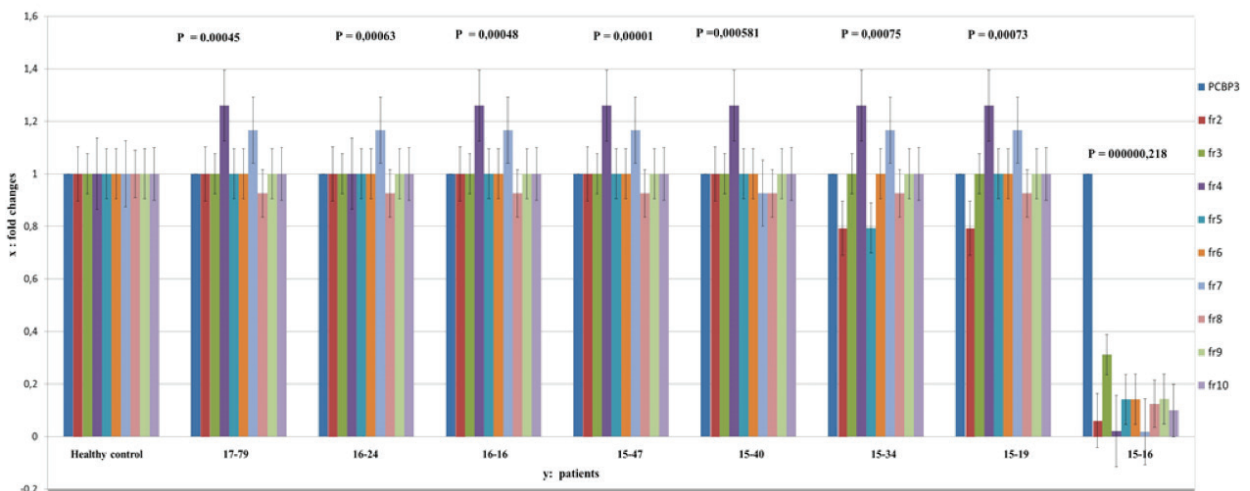


Figure 2: Graphical representation showing the comparison of the fold changes of each fragment for each patient. P values and fold changes were controlled three times. (x: fold changes y: patients *SLC2A1*: target gene, *PCBP3*: reference gene, control: a pool of six (three women-three men) unaffected individuals, fr: fragment).

relates with the haplo insufficiency of pathogenic variations in development of GLUT1-DS.

Interpretation of CNVs in *SLC2A1*

After identifying two novel pathogenic variations, 7 available patients out of 10 were analyzed for CNV events throughout the *SLC2A1* gene, using a real time qPCR approach. The result showed that only one patient (E15-16) was found to have a decreased CNV value in all *SLC2A1* fragments analyzed (Figure 2). The possible loss of the allele carrying the *SLC2A1* gene is again compatible with a null effect, which may result in GLUT1-DS phenotype through haplo insufficiency.

Interpretation of *SLC2A1* Gene Expression

SLC2A1 mRNA level analysis for E11-169 and her family showed reduced expression for the index patient compared to her parents. RT-qPCR measurements indicated statistically significant decrease in the *SLC2A1* gene expression compared to non-carrier healthy parents ($p=0,0002$). Triplicate mRNA measurements revealed a mean of 49% decrease in the transcript level for the affected patient (Figure 3). Possible mechanism for complete degradation of the mutated allele can be explained by non-sense mediated decay mechanism.

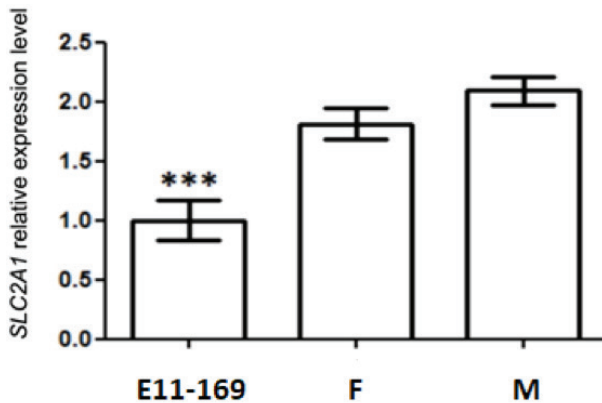


Figure 3: Triplicate mRNA measurements revealed a mean of 49% decrease in the transcript level for affected patient (E11-169) and her family (F: father, M: mother) ($p:0,0003657$).

DISCUSSION

In this study, we carried out screening of *SLC2A1* gene by means of Sanger sequencing and CNV analysis in order to detect novel variations associated with GLUT1-DS. This approach from clinics to genetics acts as a model facilitating the correct diagnosis of this disorder. Clinical diagnosis assisted with correct genetic diagnosis is the first step for the development of possible treatment options. In this case, patients diagnosed with GLUT1-DS can be good candidates for a ketogenic diet, optimal antiepileptic treatment and other supportive therapies (11).

In this study, we have identified 3 potentially pathogenic variants using Sanger sequencing and quantitative analysis of *SLC2A1* coding region. All these 3 variations (frameshift, splice region and gene copy loss) are loss of function alleles and accordingly they lead to null alleles and exert their effect on the GLUT1-DS phenotype through haploinsufficiency.

On the other hand, negative cases of *SLC2A1* may be due to another unknown genetic defect, reversible transient glucose transport disorder or even other unidentified causes such as infectious, traumatic, certain antiepileptic drugs (phenobarbital, valproate sodium) (12).

Increased blood glucose levels due to a variety of causes may also reduce CSF / blood glucose ratio and cause misdiagnosis. For example, as a result of the study conducted by Leen et al., Only 41% of 132 people had a variation (13). By way of another example, in the study by Vuillaumier-Barrot et al., the atypical AHC case was associated with a false mutation in *SLC2A1*, indicating clinical overlap between the two pathologies (14).

Screening of other GLUT members (GLUT 2/3/4/5) associated with GLUT1 and / or functional regions of *SLC16A1*, *SLC16A3*, *SLC16A7* and *SLC16A8* genes in which MCT1, MCT2, MCT3 and MCT4 proteins are encoded may be suggested for further molecular testing for *SLC2A1* negative GLUT1DS cases.

Ethics Committee Approval: Ethics committee approval was received for this study from the Istanbul University, Istanbul Faculty of Medicine Ethics Committee.

Informed Consent: Written consent was obtained from the participants.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- Y.K., İ.K.; Data Acquisition- Ö.Ö.; Data Analysis/Interpretation- B.K, N.B.; Drafting Manuscript- S.A.U.İ.; Critical Revision of Manuscript- B.K., U.Ö.; Final Approval and Accountability- C.Ö.G., B.K., İ.K., Ö.Ö., Y.K., N.B., U.Ö., S.A.U.İ.

Conflict of Interest: Authors declared no conflict of interest.

Financial Disclosure: This work was supported by the grant of Scientific Research Projects Coordination Unit of Istanbul University (Project No: TYL-2017-26212).

Etik Komite Onayı: Bu çalışma için etik komite onayı İstanbul Üniversitesi İstanbul Tıp Fakültesi Etik Kurulundan alınmıştır.

Bilgilendirilmiş Onam: Katılımcılardan bilgilendirilmiş onam alınmıştır.

Hakem Değerlendirmesi: Dış bağımsız.

Yazar Katkıları: Çalışma Konsepti/Tasarım- Y.K., İ.K.; Veri Toplama- Ö.Ö.; Veri Analizi/Yorumlama- B.K., N.B.; Yazı Taslağı- S.A.U.İ.; İçeriğin Eleştirel İncelemesi- B.K., U.Ö.; Son Onay ve Sorumluluk- C.Ö.G., B.K., İ.K., Ö.Ö., Y.K., N.B., U.Ö., S.A.U.İ.

Çıkar Çatışması: Yazarlar çıkar çatışması beyan etmemişlerdir.

Finansal Destek: Bu çalışma İstanbul Üniversitesi Bilimsel Araştırma Projeleri Koordinasyon Birimi hibesi ile desteklenmiştir (Proje No: TYL-2017-26212).

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