

Novel Nonsense *p.C522X* Mutation in *SLC5A2* Gene of a Turkish Family with Familial Renal Glucosuria: A Molecular Case Report

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Abstract

Background: Familial renal glucosuria (FRG) is a rare renal tubular disorder which has recently been shown to result from mutations in *SLC5A2* gene, encoding the kidney-specific low-affinity/high-capacity Na⁺/glucose cotransporter protein, *SGLT2*. The vast majority of FRG cases were shown with the confirmation of *SLC5A2* mutations with larger numbers of samples from various groups. Here we present a Turkish family of mild glucosuria, with nonsense *p.C522X* mutation in *SLC5A2* gene.

Methods: DNA Sequencing analysis was performed for all the affected probands and the unaffected individuals.

Results: Except for the father, the children and the mother were found out to have *p.C522X* mutation in *SLC5A2* gene together with the divergent clinical implications.

Conclusions: Rather it was found to be a sample of recessive or dominant type of renal glucosuria, the case indicated here was suggested as a model for autosomal co-dominant trait with complete penetrance and variable expressivity.

Keywords: Familial Renal Glucosuria (FRG); *SLC5A2*; Nonsense mutation; DNA cycle sequencing

Introduction

Renal glucosuria (OMIM233100) is a proximal tubular glucose transport disorder causing abnormal urinary glucose excretion with normal blood glucose levels and without proximal tubular dysfunction or morphological renal anomalies. Glucose is filtered from the blood at the glomerulus and then reabsorbed in the S1 segment of the proximal tubule via specific apical glucose transporters in kidney, the central regulators of plasma glucose levels [1]. If the filtered amount of glucose increases, glucose reabsorption reaches saturation. After passing the threshold when transporters fully saturate, the amount of reabsorbed glucose does not increase, and excretes in the urine; finally degradation of normal conditions comes out [2].

Familial renal glucosuria (FRG) (OMIM 233100) is found out to be a rare renal tubular disorder caused by mutations within the *SLC5A2* gene, the Na⁺/glucose cotransporter family [3,4]. Renal glucose reabsorption is mediated by luminal Na⁺/glucose cotransporters (*SGLTs*) and basolateral facilitative glucose transporters (*GLUTs*) in epithelial cells lining the jejunum and proximal renal tubules, which are Na⁺/glucose cotransporters concentrating glucose inside cells using the transmembrane electrochemical potential of Na⁺ [5]. To date, several nucleotide changes were shown in this gene to cause FRG. Here we report a Turkish family with an inherited novel nonsense mutation in *SLC5A2* gene (*p.C522X*) clinically thought to cause FRG.

Materials and Methods

General criteria

The study was approved by the Ethical Committee of Ege University Faculty of Medicine. Probands were informed about the study and signed (by the patient or by his/her parent) an informed

consent according to the recommendations of the declaration of Helsinki for investigation with human subjects.

Patients and control subjects

A Turkish family with two children having suspicious renal glucosuria diagnosis and 50 chromosomes from 25 healthy individuals were enrolled into the study. None of the control subjects had any signs of a generalized tubular dysfunction or of any other type of renal disease. Urinary glucose concentration was repeatedly determined by dipstick before it was quantified by the glucose oxidase method in aliquots of 24-h urine collected on a free diet in which normal range was accepted as 0.13 to 0.32 g/1.73 m² per d [0.72 to 1.8 mmol/1.73m² per d] [6].

Genomic DNA preparation and quantitation

Genomic DNA (gDNA) from 2 ml of peripheral blood samples which were collected into ethylenediaminetetraacetic acid (EDTA)-anticoagulated tubes by the standard venipuncture method was extracted using the QIAmp blood DNA Isolation kit following manufacturer's instructions. DNA concentration was determined by using Thermo Scientific Nanodrop apparatus.

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Case	Ph	Density	Osmolality	Glucose	Protein	Microscopy	24 hour Urine volume	24 hour protein	TPR	FENa	FEK	Ca/Cr	Ca excretion
			Mosm/L	mg/dl	mg/dl		ml/d	mg/m ² /h					mg/kg/d
Patient 1	6.5	1015	967	1000	Negative	Normal	1100	2.1	92	0.4	2.4	0.16	2.1
Patient 2	6	1010	804	1000	Negative	Normal	Spot	0.18	95	0.2	5.7	0.18	ND
Mother	5	1030	862	300	Negative	Normal	1500	2.5	94	0.6	4	0.09	0.8
Father	5	1025	922	0	Negative	Normal	1200	1.2	91	0.4	3.2	0.12	1.2

Table 1: Demographic features of the probands showing tubulary function analysis.

Family member	Glucose excretion (g/1.73 m ² /24 h)	Region	Nucleotide Change; Allele 1	Nucleotide Change; Allele 2	Mutation State	Mutation Type	Amino Acid change
Patient 1	5.8	Exon 12	c.1566C>A (TGC/TGA)	Wt	Heterozygous	Nonsense	p.C522X
Patient 2	5.2	Exon 12	c.1566C>A (TGC/TGA)	Wt	Heterozygous	Nonsense	p.C522X
Mother	2.0	Exon 12	c.1566C>A (TGC/TGA)	Wt	Heterozygous	Nonsense	p.C522X
Father	0.11	Exon 12	Wt	Wt	-	-	-

Table 2: Mutation analysis and glucose excretion in affected individuals.

Molecular analysis

Polymerase chain reaction and DNA sequencing analysis: Molecular analysis was carried out on genomic DNA extracted from EDTA anticoagulated venous blood using QiAamp DNA Blood Mini kit (QIAGEN GmbH, Hilden Germany) according to manufacturer's instructions. The diagnostic strategy was based on screening the 14 entire coding exons and adjacent intronic segments of *SLC5A2* gene. For the studied *SLC5A2* gene, the genbank data were as follows: NCBI Reference Sequence NG_012892.1 RefSeqGene; NC_000016.9; (NM_003041.3; NP_003032.1); definition, homo sapiens *SLC5A2* (solute carrier family 5 (sodium/glucose cotransporter), member 2-unique isoform; 2279 bp mRNA and 672 amino acid sequence length. All 14 exons of *SLC5A2* gene were amplified by polymerase chain reaction (PCR) using flanking intronic primers, designed by ExonPrimer programme. All synthetic oligonucleotide primers synthesized and purchased by Invitrogen Ltd. (Paisley, UK) as the HPLC purification grade (primer sequences are available upon request).

PCR amplification reactions were performed using 100 ng of human gDNA. PCR amplification was carried out on Corbett Palm-Cycler gradient thermal cycler (Corbett Life Science, Australia) in a 25µl reaction mixture in 0.2 ml thin-wall PCR strip tubes containing 1µl genomic DNA solution, 1.0 U Platinum TAQ with Enhancer Buffer (Invitrogen Ltd. Paisley, UK), 50µmol/l each of the dGTP, dATP, dTTP and dCTP, 5 pmol each forward and reverse primers. The cycling conditions comprised a hot start at 95°C for 10 min, followed by 35 amplification cycles at gradient programme. Before cycle sequencing reactions, the amplified PCR products were purified using ExoSap-IT PCR purification Kit (Amersham Life Science, USB). Cycle sequencing PCR was performed with BigDye Terminator v.3.1 kit as manufacturer's instructions. Cycle sequencing PCR products, after purification with BigDyeXT Terminator kit, were analyzed in ABI 3100 Genetic Analyser System. DNA sequencing was performed bidirectionally. For sequence evaluation, SeqScape 2.0 sequencing analysis software was used.

Results

Case report and clinical characterization

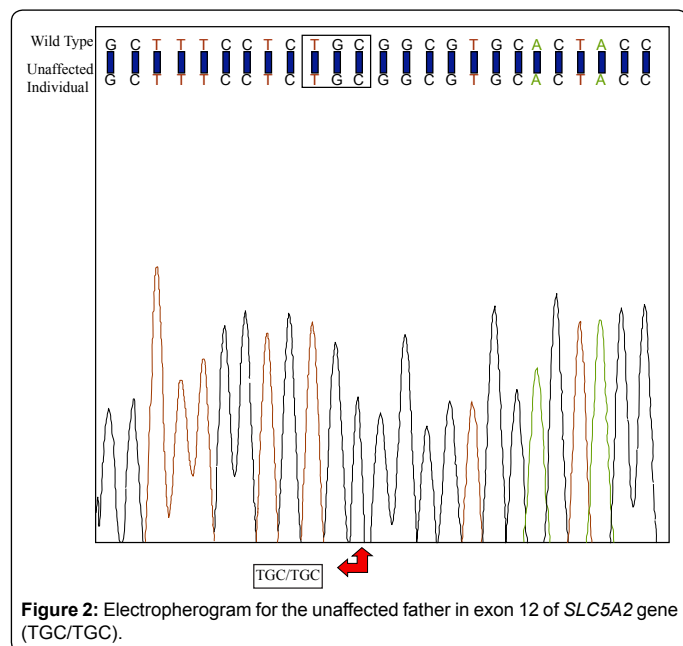
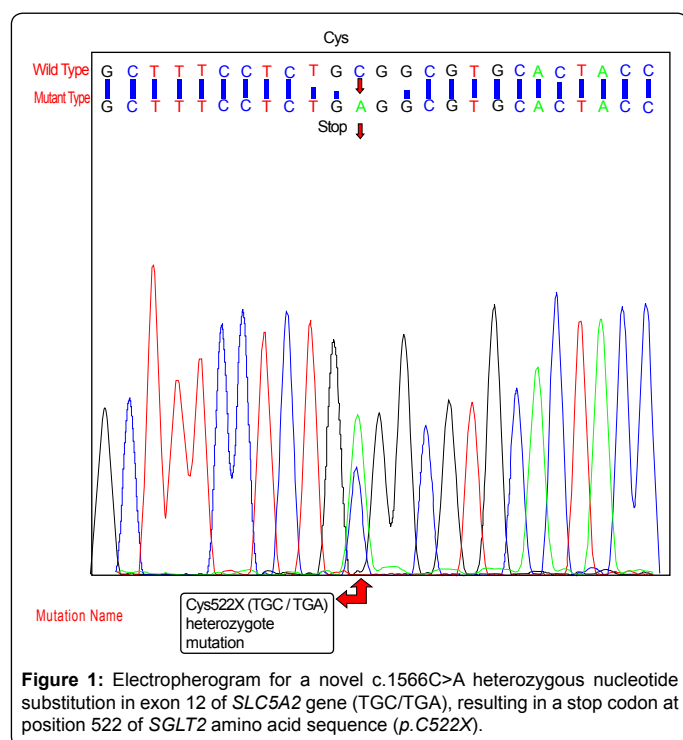
The 4.5-year-old girl (the first child of the family) was taken to Child Hospital at Ege University School of Medicine by her family for routine control. Detailed urine tests were performed in either spot urine or 24h collected urine analysis (Table 1, Table 2). Her urine dipstick analysis showed 1000 mg/dl glucose and 24 h glucose

excretion was 5.8 g/1.73 m²/24 h without other anomalies in urine. She was in normal growth and percentiles (weight: 15.5 kg and, height: 107 cm). Mental development was in normal limits. She had no any active complaints and no determined allergy. She had normal blood biochemistry results including normal blood glucose levels of 96 mg/dL. Polyuria, polydipsia and proteinuria were not seen. Kidney function tests, phosphate, protein creatine, absorption of free amino acids and urine acidification with carbohydrate metabolism, storage, usage and insulin secretion were all normal. None of them had any pathology related to kidney function diagnosis with the exception of glucosuria. It was confirmed that the excreted sugar was glucose excluding pentosuria, fructosuria, sucrosuria, maltosuria, galactosuria, and lactosuria (the renal abnormality is specific to only glucose, not to other monosaccharides). Relative to assessment of osteomyelitis and growth retardation which this mutation accompany with, Vit-D and PTH have been searched and normal line clinical evidence was determined. Bone age of the case was in accordance with the chronological age. With normal USG assessment of the case, polyclinic observation has been decided. There was no tubular dysfunction according to tubulary tests including FENa, FEK, TPR, and urine concentration. Ca/Cr ratio and urine protein levels were found to be in normal ranges (Table 1). Endocrinological profiles including Hemoglobine A_{1c}, parathormone, thyroid function tests, and VitD were also in normal ranges. She was accepted as renal glucosuria. Other family members including the ten-months-old sister, mother, and father were examined. Her mother and ten-months-old sister were also found to have glucosuria in levels of 300 mg/dl and 1000 mg/dl, and 24 h glucose excretions were 2.0 and 5.2 g/1.73 m²/24 h, respectively. Father's urine analysis showed no glucosuria.

Results of molecular analysis

Mutation analysis in the 4.5-year-old proband revealed a novel 1566C>A heterozygous sequence change in exon 12 of *SLC5A2* gene (TGC/TGA), resulting in stop codon at position 522 of *SGLT2* amino acid sequence (*p.C522X*) (Figure 1, Figure 2 and Figure 3). Following this result, the sister, father, and mother had taken to mutation screening of *SLC5A2* gene by DNA sequencing analysis system. C522X nonsense mutation was found in the mother and the sister, and the father did not have the mutation.

In the DNA sequencing analysis, the heterozygous mutation (TGC→TGA; C to A transition at position 1566) was not identified in 50 control chromosomes. The identified mutation, *p.C522X*, is a nonsense truncating mutation that would cause premature



termination of translation. This would result in a truncated SGLT2 protein lacking the last 150 amino acids which was localized close to the carboxyl terminus of the protein.

Discussion

Familial renal glucosuria (FRG, McKusick 233100) is an inherited disorder of proximal tubular glucose transport characterized by persistent glucosuria in the presence of normal serum glucose concentration and absence of any tubular nonfunctionality. Santer et al reported the major candidate gene *SLC5A2* for FRG, chromosomally located in 16p12-p11 which has 14 exons and a unique transcript of 672 amino acid sequence length encoding the low affinity sodium

glucose cotransporter *SGLT2* protein [7]. Subsequently, the vast majority of FRG cases were shown with the confirmation of *SLC5A2* mutations in various exons of the gene and from various groups [7-12]. To date, in renal glucosuria patients, mutations belonging to exon 12 had first been reported by Calado et al. [13] including also nonsense mutations. In a case report of Van den Heuvel et al. [12], a Turkish patient with congenital isolated renal glucosuria having a homozygous nonsense mutation in exon 11 of the *SLC5A2* gene leading to the formation of a truncated cotransporter has been reported. In that study, both parents and the brother of the child, all three without renal glucosuria, were heterozygous for the nonsense mutation. Heterozygous individuals were reported to have only mild glucose excretion (2.7-6.5 g/1.73 m²/24 h) whereas homozygous or compound heterozygous patients had severe glucosuria ranging from 10 to 86.5 g/1.73 m²/24 h. Mutation analysis in the probands of our study revealed a novel heterozygote nonsense mutation in exon 12 of *SLC5A2* gene (TGC/TGA) at the position of 1566 cDNA resulting in *p.C522X* nonsense mutation which was not found in the 50 healthy Turkish alleles. For the probands in this study, thought as mild glucosuria, the amount of glucose excreted ranged from 2.0 to 5.8 g/1.73 m²/24 h, reflecting the heterozygous character of the mutation. Since the cases presented here were seen as mild glucosuria, basal plasma renin activity and serum aldosterone concentrations were not determined.

In this study, although the mother and the two daughters share the same nonsense mutation, their clinical implications had some obvious differences relative to glucose levels. The clinical divergent implications seen in the family members brought some challenge with the autosomal recessive inheritance pattern of the disease. Also, in Calado's report, one case having heterozygous *p.N654S* sequence variation, referred to as a polymorphism, reported as having mild glucosuria [13]. In this case, rather it was found to be a sample of recessive or dominant type of renal glucosuria, the case indicated here was seen as an autosomal co-dominant trait with complete penetrance and variable expressivity. Besides, it may be thought that a polygenic inheritance pattern (involving other genetic or nongenetic factors-environmental, physical or social factors, sample size of patients, molecular genetic analysis procedure, involvement of noncoding regions as well as the coding regions in the molecular analysis) can be seen in renal glucosuria with the contribution of relative modifying genes leading genetic heterogeneity in the disorder. Modifier genes, other possible sodium glucose co-transporter members of *SLC5* family comprising over 230 members involving 11 members of the human genome, may have a role in defining the particular phenotype and account for the incomplete penetrance defined in FRG. Genetic heterogeneity may be caused by other cotransporters which may

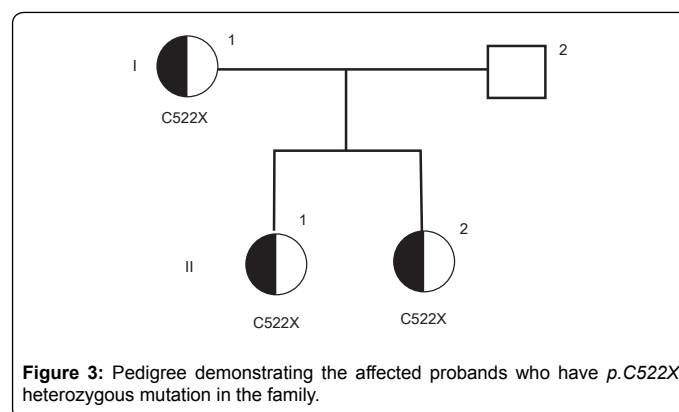


Figure 3: Pedigree demonstrating the affected probands who have *p.C522X* heterozygous mutation in the family.

be entitled as modifier genes that constitutes the incomplete penetrance seen in FRG. The results of this study are in accordance with earlier clinical and molecular observations dealing with the inheritance pattern that heterozygous mutations are associated with mild glucosuria (2,7-6,5 g/1.73 m²/24h). Despite the significant role of *SLC5A2*, other genes may be involved in renal glucose transport to varying degrees and may be mutated in patients with renal glucosuria. Especially in family case reports of hereditary disorders, the divergence of the phenotype strongly suggest the impact of other genes in occurrence of the current disease. Possible mechanisms for the unexplained transcriptional regulation of *SGLTs* and the genetic heterogeneity seen in FRG caused by the presence of other genes and modifier genes. For *SGLT1*, role of protein kinases were shown, and suggested that sequence differences in *SGLTs* could effect the regulation of trafficking by protein kinases [14]. Five transcription factors (HNF-1 alpha, HNF-1 beta, HNF-4alpha, IPF-1, and NEURO-D1) produce different forms of diabetes [15-17], and a defect of HNF-1 α cause maturity-onset diabetes in the young type 3, with diminished renal threshold for glucose, which was not present in the probands of this study. Also, hepatic nuclear factor (HNF)-1 α was found out to regulate the transcription of mouse *SGLT2* [18], and for human *SGLT1*, critical role for the HNF-1 and Sp1 (specificity protein 1) multigene members in enhancing the basal level of its transcription (the binding domain for this transcription factor) in Caco-2 cells was reported [19]. Kekuda et al. [20] reported that Sp1 together with HNF1 transcription factor regulate *SGLT1* transcription in the chronically inflamed intestine. Also, Tabatabai et al. [21] showed diabetes related changes in mRNA expressions of kidney glucose transporters *SGLT1* and 2 in Zucker rats. Furthermore, mutations in other genes, having strong genetic linkage as shown for the *HLA* locus and *GLYS1* in renal glucosuria [22], and the entire members of *SGLT* and *GLUT* family should be considered as possible candidates.

Regarding the reported high similarity of the primary sequences and secondary structure of *SGLT1* and *SGLT2*, Santer et al. [7] showed 58% amino acid identity between *SGLT1* and *SGLT2* protein, and a higher degree of homology was also observed in central domains of the polypeptide. Though there is little information available about *SGLT2* due to its low level of expression, the general secondary structure model shows 14 transmembrane helices (TMH) with both the NH₂ and COOH termini facing the extracellular region [23]. Panayotova-Heiermann et al. [24] reported that while sugar-binding/translocation to Na⁺/glucose cotransporters was determined by the COOH-terminal domain (sugar affinity and selectivity; residues 407–662 containing TMHs 10–14, referred to as C5), Na⁺ binding/coupling is determined by the NH₂-terminal domain of the protein, and conformational change in the protein allowing sugar binding and translocation occurs. TMH 5 and 6 were shown in 8 Å of TMH 10–11 [25]. In the expression studies of the same group, truncated protein containing residues 407–662 (C5) were shown as a sugar uniporter [26,27]. However, in 2001, Wright et al. [28] reported that deletion or missing of TMH 14 did not disturb sugar transport, meaning only TMHs 10–13 lie in close proximity constituting the sugar translocation pathway through *SGLT1*. It was reported that in *SGLTs* amino terminus (residues 1–27), two hydrophilic loops located in the center (residues 229–271), and the carboxyl terminus (residues 548–644) constitute the nonconserved polar residues, and possibly responsible for the charge movement and/or substrate specificity differences. Panayotova-Heiermann et al. [24] reported that recognition/transport domain of organic substrate is mediated by interactions distal to amino acid 380, while cation binding is determined by interactions arising from the amino- and carboxyl-

terminal halves of the transporters, and found out the largest loop between M13/M14 involving most of the differently charged amino acid substitutions any of which could contribute to the organic substrate binding.

SGLT2 nonsense, frameshift, and missense mutations are predicted to result in disturbed affinity for the specific substrate due to the absence of transmembranous domains 10 to 13 essential for sugar binding/transport, (when the 14th helix deleted, only four helices, 10–13, were sufficient for sugar transport pathway), and thus leading to mild glucosuria [27]. In Santer et al. [11] study, patient with P324fs347X homozygous mutation leading to a truncating protein, and predicted to have only the seven N-terminally located transmembranous domains without the missing TMH 8-14 domains demonstrated a daily glucose excretion in excess of 100 g/1.73 m² per d. Obviously, it was a nonfunctional glucose transporter underscoring the lack of glucose reabsorption due to the absence of the transmembranous domains 10 to 13 which were postulated to have a role for sugar binding/translocation [24,26,28]. Interestingly, another patient in that study with homozygous W440X nonsense truncating mutation showed lower glucose excretion (68.7 g/1.73 m² per d;), there was a challenge and suggested alternative mechanisms for glucose reabsorption. The same group also documented that all heterozygous individuals had not increased glucose excretion, and only some had mild glucosuria, suggesting a place for possible genetic or nongenetic factors to be present accompanying the heterozygous *SGLT2* mutations which were expected to result in mild glucosuria. Therefore it would be more convenient to suggest a codominant trait with expressivity.

Directed mutagenesis studies provided the identification of amino acid residues important for substrate and inhibitor binding. In Wright's study, a compound heterozygous patient (*SGLT1* R499H-helix 12/R379X) with glucose-galactose malabsorption was shown with severe clinical course, and this mutant was demonstrated to result in a significant decrease of sugar affinity and trafficking to the membrane in the functional expression studies performed in oocytes. The replacing of this residue with cysteine was suggested to result in restored trafficking and functional sugar transport [29]. Steel and Hediger [30] studied A304V and R499H-helix 12 mutations and determined impaired trafficking of the cotransporters to the plasma membrane, as in accordance with the results of Martin et al. [31] who suggested a role for polar and charged residues in the retention of protein conformation due to electrostatic interactions. Also, *SGLT1* R427A mutation was shown in membrane targeting due to the accumulation of the mutant protein beneath the membrane surface. Since, Q457A-helix 11 showed reduced sugar transport and R499H was along with an increase in K_m, these residues were thought to lie close to the sugar binding site (COOH). *SGLT1* N176A was reported to effect only the presteady state kinetics and not the sugar-induced steady state [32]. K321A-helix 7 mutants decreased the affinity for Na⁺ ions, without changes in sugar affinity (only secondary effects on sugar binding to the protein) [33]. N176A and K321A were located in the amino-terminal half of *SGLT1* and did not substantially effect sugar binding, in contrary to R499H, found out in a glucose/galactose malabsorption patient, which was localized close to the carboxyl terminus of the protein [31], and proved a decrease in the apparent affinity for sugar without affecting Na⁺. Althoff et al. [34] reported Thr/459 and Ser/460 in the *SGLT* from shark located in transmembrane helix 11 indicating sugar translocation pathway [26]. Kasch [35] found Cys351 and Cys361 (*SGLT1* and *SGLT2*) in the extracellular loop 8 defining sugar affinity of the transporter.

Wright [36] described *p.Q167fsX186* mutation leading to a truncated protein in the absence of the last 505 C-terminal amino acids, the region that contains the consensus N-linked glucosylation sites and transmembrane domains 10–13, lacks the sugar translocation path. Lostao et al. [37] described reduced trafficking and amount of C5 molecules in the plasma membrane because of a single residue (R427A) in *SGLT1*. According to these previous reports, when considering the highly identical membrane topology of *SGLT1* with *SGLT2*, *p.C522X* nonsense truncating mutation are predicted to locate in the extracellular loop 13 and C5 (amino acids 407–672, helices 10–13) lying in close proximity to the sugar binding site (COOH), and lacking the last 150 amino acids/helix 14/TMH13-14, and are thought to effect sugar affinity (without any effect on Na⁺) and trafficking of the *SGLT2* transporter. It was reported that TMH10-13 were important for sugar affinity except for TMH14. In this study, it is obvious from the daily glucose excretions in the probands that the absence of TMH13 without 10, 11, and 12, was sufficient to give the evidence of typical isolated renal glucosuria phenotype. However, the argument that a mutation affects a highly conserved functionally important region of a gene/protein is not very relevant in the case of a nonsense mutation. Truncation of the protein, will lead to loss of other regions distal to the mutation. According to above previous reports, missense and nonsense *SLC5* mutations leads to disorder because of the missing expression of such proteins within the cell membrane, and the problem is not the functional loss. Furthermore, analysis of this newly found mutation, in order to evaluate the potential pathogenicity, was not carried out by the PolyPhen software [38] because it was already a nonsense mutation whose pathogenic significance is obvious and predicted to be possibly damaging. However, in FRG, future studies are required to confirm critical domain functions of *SGLT2* which is currently a drug target for the treatment of type II diabetes [39].

Nevertheless, great sequence and structure homology of *SGLTs* were reported and it can be surely thought that disturbed trafficking shown to play a major role not only for *SGLT1* mutations but also for *SGLT2* [31]. In this study, we were not able to perform expression patterns of *SGLT2* mutations. However, human *SGLT2* expression in heterologous systems, unlike *SGLT1* and *SGLT3*, was reported to be very poor in oocytes and COS-7 cells [36,40]. Santer et al. [7] expression studies worked on *Xenopus laevis* oocytes and in isolated vesicles with perfect expression patterns with *SGLT1* and other transporters, but not with *SGLT2*. So, the mechanism is not yet obvious for *SGLT2* mutations in the impairment of glucose transport.

In conclusion, this data is the first direct evidence of an etiologic role for the sodium/glucose cotransporter type 2 in the pathogenesis of renal glucosuria in a Turkish family. The detection of certain *SGLT2* mutations predicted to be nonfunctional correlates with the lack of glucose reabsorption and proves the critical requirement for *SGLT2* in renal tubular glucose transport.

Acknowledgements

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