Disruption of urate transport in familial renal glucosuria and report on SGLT2 expression in normal and pathological kidney

Alteração do transporte de ácido úrico na Glicosúria Renal Familiar e expressão de SGLT2 no rim normal e patológico

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ABSTRACT

Familial renal glucosuria (FRG) is a rare co-dominantly inherited benign phenotype characterized by the presence of glucose in the urine. It is caused by mutations in the SLC5A2 gene that encodes SGLT2, a Na+-glucose co-transporter. The purpose of our current work was twofold: to characterize the molecular and phenotype findings of an FRG cohort and, in addition, to detail the SGLT2 expression in the adult human kidney. The phenotype of FRG pedigrees was evaluated using direct sequencing for the identification of sequence variations in the SLC5A2 gene. The expression of SGLT2 in the adult human kidney was studied by immunofluorescence on kidney biopsy specimens. In the absence of renal biopsies from FRG individuals, and in order to evaluate the potential disruption of SGLT2 expression in a glucosuric nephropathy, we have selected cases of nucleoside analogues induced proximal tubular toxicity. We identified six novel SLC5A2 mutations in six FRG pedigrees and described the occurrence of hyperuricosuria associated with hypouricaemia in the two probands with the most severe phenotypes. Histopathological studies proved that SGLT2 is localized to the brush-border of the proximal tubular epithelia cell and that this normal pattern was found to be disrupted in cases of nucleoside analogues induced tubulopathy. We present six novel SLC5A2 mutations, further contributing to the allelic heterogeneity in FRG, and identified hyperuricosuria and hypouricaemia as part of the FRG phenotype. SGLT2 is localized to the brush-border of the proximal tubule in the adult human normal kidney, and aberrant expression of the co-transporter may underlie the glucosuria seen with the use of nucleoside analogues.

Key words: glucosuria; proximal tubule; SGLT2 expression; uric acid.
INTRODUCTION

Familial renal glucosuria (FRG) is characterized by the presence of glucose in the urine in the absence of diabetes mellitus or generalized proximal tubular dysfunction. Mutations in the SLC5A2 gene, positioned in 16p11.2 and encoding SGLT2, the Na+-coupled glucose transporter that accounts for the bulk of glucose reabsorption in the renal proximal tubule, are responsible for the large majority of FRG pedigrees. Most of these mutations are private and usually involve missense alleles, although nonsense, small deletions (both in-frame and frameshift) and splice site mutations have been reported. Even though an increasing number of FRG cohorts is being reported, patients presenting with severe forms of FRG with glucosuria in excess of 50 g/1.73m²/24h are distinctly rare.

Two members of the Na+-coupled glucose transporter family, SGLT2 and (to a lesser extent) SGLT1, are responsible for the secondary active uptake of glucose from the proximal lumen at the apical side of the epithelia. Once within the cell, glucose is returned to the systemic circulation by facilitative transport mediated by GLUT1 and 26.

Renal tubular glucose transport has been pointed out as a target in the treatment of type 2 diabetes mellitus (T2DM) and several SGLT2 inhibitors (SGLT2i) are now awaiting FDA approval. In a previous report, we anticipated that these compounds were likely to have a diuretic effect, based on the findings of moderate volume contraction with secondary activation of the renin-angiotensin-aldosterone system in severe FRG phenotypes. This was later ascertained in clinical trials with SGLT2i. Our group performs routine molecular characterization of FRG pedigrees and, whenever possible, phenotypic evaluation of such individuals. We now report the molecular and clinical findings of six pedigrees displaying novel SLC5A2 alleles. For two of these probands, glucosuria was in the range of the urinary glucose excretion (UGE) induced by pharmacological inhibition of SGLT2. One interesting and consistent...
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observation with the administration of SGLT2i, is the
almost dose-proportional decrease in serum uric acid
levels7,8, which is paradoxical in light of the previ-
ously mentioned diuretic effect. We have, therefore,
evaluated severe FRG phenotypes regarding serum
and urinary uric acid levels. In addition, we assessed
SGLT2 protein expression in adult normal human
kidney and validated an FITC-immunofluorescence
technique for paraffin-embedded kidney biopsy
samples, with the purpose of detailing the topologi-
ical location of SGLT2. Both techniques were per-
formed using an available commercial antibody. In
the absence of renal specimens from FRG individuals,
we have searched for aberrant SGLT2 expression in
renal biopsies of patients with nucleoside analogues
induced proximal tubular toxicity and glucosuria.

SUBJECTS AND METHODS

Phenotype evaluation and mutation analysis
of FRG pedigrees

This study was part of the medical evaluation for
glucosuria. After obtaining informed consent from
participating individuals or their legal guardians,
individuals were enrolled. Mutation analysis was
performed as previously reported9, but for exons 10
to 12, a single amplicon of 978 bp was PCR ampli-
fied and directly sequenced, using the following
forward 5'-CTCGTGAGCTCATGC-3' and reverse
5'-CCAACCCCTCAGTCGAGAAAT-3' primers. Only indi-
viduals bearing SLC5A2 mutations not previously
described were included in this report.

One hundred control chromosomes were assayed
for the presence of the missense mutations identified,
excluding them as common polymorphisms. We used
direct sequencing of exons 5 and 12, for the c.571
A>C and c.1475 G>T alleles, respectively. In the case
of the c.1343 A>T transversion, a new TspRI restriction
site was inserted within the 978 bp exon 10-12 ampli-
con, therefore enabling easy testing by PCR-RFLP of
affected members and control chromosomes.

Immunohistology of kidney biopsies

SGLT2 detection by immunofluorescence was per-
formed in five formalin-fixed and paraffin-embedded
kidney biopsies with no detectable histopathological
changes. Two additional sample of known proximal
urinary tract toxicity with glucosuria induced by tenofovir
or adefovir were also included. Heat-induced epitope
retrieval was applied to 3μm sections with Target
Retrieval Solution pH 9 (ref. S2367, Dako) at 95-99°C
for 20 min. Incubations with primary goat polyclonal
antibody anti-human SGLT2 C-terminal domain, from
residues 590-640 (ref sc-47401, from Santa Cruz Bio-
technology (1:5) and a secondary antibody donkey
anti-goat IgG-FITC (1:20) – ref sc-2420, Santa Cruz
Biotechnology – were carried out for 60’ at room
temperature.

RESULTS

Phenotype evaluation and mutational analysis

We identified six novel alleles in 13 individuals,
from six different pedigrees. The available clinical
data is detailed in Table I. Relatives were screened
for glucosuria by dipstick urine analysis and, when-
ever possible, quantified by a 24h urine collection.
All affected individuals had normal renal function.
Two heterozygous, one compound heterozygous and
three homozygous probands were identified. The
two probands with the most severe phenotypes, with
UGE of 87.8 and 43.7 g/1.73m2/24h – individuals I.1
of pedigree 1 and II.1 of pedigree 2 – also had
hyperuricosuria: excreting 1242 and 990 mg/1.73m2/24h
(reference values 250-750), with an urinary uric acid
(mg)/Kg of body weight of 19.1 and 21.5 (reference
values in a healthy population: 7.6 ± 3.75 [adolescents]
and 7 ± 1.6 [adults] 10, respectively. For proband of
pedigree 1 (the most severe of our cohort) this was
clearly associated with hypouricaemia of 1.9 mg/dl
(reference range: 2.5-7). Proband II.1 of family 2,
although having a serum uric acid of 3.5 mg/dl at
the time of evaluation, by the age of eight years the
reported value was of 2.4 mg/dl.

Three mutated alleles are missense substitutions:
p.T191P, p.Q448L and p.G492V. All represent non-
-conservative substitutions (with the exception of
Val for Gly, in that both are non-polar amino acids)
affecting highly conserved residues among SGLT
members (Fig. 1). Their putative pathogenicity was
further assessed in silico using the bioinformatic
algorithm Polyphen and, as such, all were predicted
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Table 1
Phenotype evaluation and mutational analysis in affected individuals and their relatives.

<table>
<thead>
<tr>
<th>Family members</th>
<th>Country of origin</th>
<th>Weight (Kg)</th>
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<th>Glucose excretion (g/1.73m²/24h)</th>
<th>Plasma glucose (mg/dl)</th>
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<tr>
<td>Family 1</td>
<td>Portugal</td>
<td>65</td>
<td>167</td>
<td>87.8</td>
<td>74</td>
<td>5.4</td>
<td>1.9</td>
<td>1242</td>
<td>c.1030_1057del.p.V346fsX17</td>
<td>c.1030_1057del.p.V346fsX17</td>
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<td>Family 2</td>
<td>Costa</td>
<td>na</td>
<td>na</td>
<td>46</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
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<tr>
<td>Family 3</td>
<td>Turkey</td>
<td>25</td>
<td>126</td>
<td>43.7</td>
<td>96</td>
<td>5.9</td>
<td>3.5</td>
<td>990</td>
<td>c.1946 G→p.W649X</td>
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<tr>
<td>Family 4</td>
<td>Turkey</td>
<td>31</td>
<td>140</td>
<td>25</td>
<td>50</td>
<td>5.5</td>
<td>4.4</td>
<td>44</td>
<td>c.1566C→p.C522X</td>
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<tr>
<td>Family 5</td>
<td>Turkey</td>
<td>58</td>
<td>155</td>
<td>144</td>
<td>71</td>
<td>4.1</td>
<td>3.4</td>
<td>34</td>
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</tr>
<tr>
<td>Family 6</td>
<td>Portugal</td>
<td>82</td>
<td>174</td>
<td>45</td>
<td>114</td>
<td>5.4</td>
<td>3.6</td>
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Family 3 displays a unique genetic feature. The proband, II.2, is a 14-year-old girl born of a consanguineous marriage. Yet, her severe phenotype is not related to consanguinity but to compound heterozygosity: in addition to the ancestral p.Q448L mutation, shared by both parents, she displays the donor splice

Figure 1
Sequence alignments: Multiple alignments of SGLT2 proteins from different species and comparison to human SGLT1 and SGLT3. The missense alleles of the current cohort are represented on top. The highly conserved residues are highlighted in grey.

to be probably damaging. In addition, these sequence variations were not detected in 100 control chromosomes and were, therefore, excluded as common polymorphisms. There are three truncating mutations, including two non-sense, p.C522X and p.W649X and one frameshift, p.V346fsX17.

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site mutation IVS7+5g>a transmitted by the father, in trans with the former allele. The remaining siblings are homozygous for the ancestral p.Q448L mutation, as anticipated in consanguineous marriages.

**SGLT2 expression in human kidney**

Immunofluorescence experiments in five kidney biopsies with no detectable histopathological changes localized SGLT2 to the brush-border of proximal tubular epithelia in normal kidney (Fig. 2A). Two patients with nucleoside analogues induced tubulopathy and glucosuria were identified in our pathological database. The first patient presented with acute renal failure after the introduction of tenofovir for his HIV condition. At renal biopsy, serum creatinine (Scr) was 7.0 mg/dl (on dialysis) and the 24h UGE of 4.9 g (175 mg/dl). The second individual had been for several months on adefovir for hepatitis B infection, before referral to our department. At evaluation, Scr was 1.7 mg/dl and the 24h UGE of 15.4 g (358 mg/dl). By

![Figure 2](image-url)

**Figure 2**

SGLT2 expression in human kidney. A) Immunofluorescence clearly localizes SGLT2 expression (white arrow) to the brush-border of proximal tubular cells in normal kidney(x400). B) Renal biopsies from patients with nucleoside analogues induced proximal tubular toxicity. Top: tenofovir case with loss of SGLT2 expression from the apical side of proximal epithelia (arrowhead)(x400). Bottom: adefovir case with significant reduction in the expression of the cotransporter, although some discontinuous pattern is still seen (arrowhead)(x400).
immunofluorescence, apical SGLT2 expression was abolished in the biopsy of first patient, while in the second case it was significantly reduced (Fig. 2B).

**DISCUSSION**

The kidney contributes to glucose homeostasis by reabsorbing at the proximal tubule virtually all of the glucose filtered by the glomerulus, in a two-step process. Firstly, glucose and Na⁺ are sequentially cotransported from the tubular lumen via secondary active transport mediated by the renal Na⁺-glucose co-transporters SGLT2 and SGLT1. Secondly, glucose is released from tubular epithelial cells into the systemic circulation by facilitative Na⁺-independent glucose transporters GLUT1 and GLUT2. SGLT2 is a low-affinity, high-capacity transporter, predominately expressed at the most proximal S1 tubular segment of the nephron. Under physiological conditions, SGLT2 reabsorbs the majority of the filtered glucose and is considered a candidate gene for FRG. Mutations in SGLT1 account for the glucose-galactose malabsorption syndrome (GGM), which is characterized by severe diarrhoea upon the introduction of glucose or galactose to the diet of infants. The different phenotypes of FRG and GGM reflect the differential expression of both co-transporters as assessed by RT-PCR: SGLT1 being expressed mainly in intestinal epithelia, while SGLT2 is mostly restricted to the proximal renal tubule. Another important difference between both transporters is the apparent difficulty in assessing SGLT2 expression by immunodetection, as compared to SGLT1. In fact, most of the work for SGLT2 was done by in-situ hybridization or RT-PCR.

This report aims at further clarifying the renal tubular glucose transport by reporting the phenotype and genotype findings of FRG pedigrees displaying novel SGLT2 alleles, particularly for those individuals with severe forms, and also by assessing SGLT2 protein expression in adult human kidney.

For the six pedigrees analysed, there are six novel SLC5A2 alleles and the IVS7+5g>a mutation already reported. According to the previously described co-dominant pattern of inheritance for FRG, those individuals homozygous or compound heterozygous for a given mutation were shown to have larger UGE, when compared to heterozygous patients. For this cohort, the previously defined 10 g/1.73m²/24h cut-off appropriately seems to apply, with the exception of proband from pedigree 2. In this individual having an unusual severe phenotype, only one mutation was characterized. In fact, a second sequence variation in intron 3 (IVS3-20g>a [data not shown]) was identified, but after testing the remaining family members, it was found to be in cis with the nonsense mutation. It is possible that the second allele might have been entirely missed, either because it resides outside the coding region or it consists on a large DNA rearrangement not amenable to detection by routine PCR based techniques. Moreover, a dominant negative effect or transheterozygosity with another gene coding for a protein also having a role in renal glucose transport, are alternative explanations.

All characterized sequence variations are expected to be deleterious for SGLT2 function: the three nonsense mutations are predicted to be pathogenic substitutions, while the p.V346fsX17, p.C522X and p.W649X mutations, expected to lead to truncated proteins at SGLT2 transmembrane domains 3, 13 and 14, respectively. The IVS7+5g>a allele of Family 3 is recurrently reported in unrelated pedigrees of different ethnic origins. Considering the significant allelic heterogeneity in FRG, with most mutations being family specific, the high recurrence for this single allele can be due to a mutational hot-spot. This pedigree represents the first instance of allelic heterogeneity in a consanguineous FRG pedigree, in that the proband is a compound heterozygous for the ancestral p.Q448L mutation, shared by both parents, and the splice site mutation in intron 7. This family furthermore illustrates the co-dominant nature of FRG, with all members being affected irrespectively of heterozygosity (mother), compound heterozygosity (father and proband) or homozygosity (remaining siblings) for the identified mutations.

Hyperuricosuria with hypouricemia is the most interesting phenotype finding in the probands presenting with the highest UGE, suggesting urinary urate over-excretion as the mechanism underlying hypouricaemia in FRG and in pharmacological SGLT2 inhibition. These observations also rule out the possibility of non-specific pharmacological inhibition of renal urate transporters like GLUT9, the facilitative glucose transporter encoded by the SLC2A9 gene, mutated in familial renal hypouricaemia and expressed at the proximal renal tubule in humans.
Interestingly, GLUT9 was also shown to exchange extracellular glucose for intracellular urate17. Two isoforms, GLUT9S (expressed at the apical side) and GLUT9L (at the baso-lateral side) act concertedly to reabsorb uric acid from the glomerular filtrate. However, in case of a high glucose concentration in the proximal tubular lumen, as seen in FRG and with SGLT2 pharmacological inhibition, the direction of urate transport could be reversed, with glucose being exchanged for urate and leading to excessive urinary excretion and secondary hypouricaemia.

We have detailed the expression of SGLT2 in human adult kidney by assessing its expression by immunofluorescence in biopsy specimens. A previous report4 had already localized SGLT2 to the apical side of proximal renal tubular epithelia in humans. Our experiments confirm that SGLT2 is, in fact, expressed at apical side of normal proximal tubular epithelia and confined to the brush-border. Importantly, its expression is distinctly absent or reduced from the apical side of the proximal tubule in the cases of antiretroviral renal toxicity, both presenting with proximal tubulopathy with glucosuria. These anticipated “negative controls” not only confirm the specificity of the antibody used, but also identify the loss for SGLT2 expression as the mechanism underlying the glucosuria induced by some nucleoside analogues.

In conclusion, we present the phenotype and genotype characterization of six FRG pedigrees displaying six novel SGLT2 mutations. The hyperuricosuria found in FRG is a plausible direct cause for the hypouricaemia in the setting of severe glucosuria, either occurring naturally -as in FRG- or induced by pharmacological inhibition of SGLT2. We theorized about GLUT9 being the transporter responsible for these observations, by means of an antipporter mechanism. Finally, we localized SGLT2 in the human adult normal kidney, to the brush-border of proximal tubular epithelia cell.

Acknowledgments

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Conflict of interest statement: None declared.

References


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