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Identification of ten novel SLC5A2 mutations and determination of the renal threshold for glucose excretion in Chinese patients with familial renal glucosuria

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ABSTRACT

Background: Familial renal glucosuria (FRG) is a rare renal tubular disorder characterized by isolated persistent glucosuria without both abnormal glucose metabolism and any signs of proximal tubular dysfunction. SLC5A2 gene mutations are responsible for most FRG cases.

Methods: Quantitative test for 24-hour urine glucose and RT_G were determined in 9 families (totaling 25 subjects). All coding regions, including intron-exon boundaries, were analyzed with PCR followed by direct sequence analysis.

Results: Ten novel mutations were identified (c.331 T > C, p.W111R; c.374T > C, p.M125T; c.394C > T, p.R132C; c.612G > C, p.Q204H; c.829C > T, p.P277S; c.880G > A, p.D294N; c.1129G > A, p.G377S; c.1194C > A, p.F398L; c.1540C > T, p.P514S and c.1573C > T, p.H525Y). c.886(-10_-31)del that is specific to Chinese population was found in 5 out of 9 families, with a mutation rate of 28% (5/18). The compound heterozygotes presented with much lower RT_G values (1.28 ± 0.10 mmol/L), compared with the carriers of heterozygous variants (5.14 ± 0.77 mmol/L) ($p < 0.01$); c.886(-10_-31)del heterozygotes had significant lower RT_G values than others (4.43 ± 0.37 *vs* 5.7 ± 0.51 mmol/L; $p < 0.01$).

Conclusions: Ten novel SLC5A2 mutations are found and c.886(-10-31)del may be a hot spot mutation in Chinese population. Compound heterozygotes had much lower RT_G values than simple heterozygotes. Mixed-meal tolerance test is a simple method for determining RT_G in FRG patients.

1. Introduction

In healthy individuals, almost all of the filtered glucose is reabsorbed from the proximal convoluted tubule to maintain blood glucose levels within a normal range. The net result of filtration and reabsorption is that no glucose appears in the urine. However, when the filtered glucose load exceeds the tubular maximum glucose reabsorptive capacity (T_mG), excess glucose spills over into urine.

Familial renal glucosuria (FRG) is a rare renal tubular disorder characterized by isolated persistent glucosuria in the absence of both abnormal glucose metabolism and any other signs of proximal tubular dysfunction [1,2]. The prevalence of FRG is approximately 1/20000 in the cohort reported by Brodehl et al. [3]. Larger numbers of studies have demonstrated that SLC5A2 gene mutations are responsible for the vast majority of FRG cases [4–6]. The SLC5A2 gene is mapped to 16p11.2 and codes for the low-affinity sodium/glucose co-transporter 2 (SGLT2) that accounts for tubular reabsorption of the bulk of the filtered glucose [7].

To date, 71 different SLC5A2 mutations have been reported in association with FRG, most of them are private. Recent studies suggested that the inheritance of FRG should be best described as codominant with incomplete penetrance [8]. Targeting renal glucose excretion through inhibiting SGLT2 is a new strategy to reduce hyperglycemia in type 2 diabetes (T2DM). Several selective inhibitors of SGLT2 have been developed, and they have shown to promote glucosuria, lower plasma glucose concentration without changing insulin levels, improve insulin sensitivity, and reduce hepatic glucose output [9,10].

In a previous research we have performed SLC5A2 gene mutation analysis in 16 Chinese patients from 8 families, and found the mutation c.886(-10_-31)del rate reached up to 37.5% which may be a hot pot mutation in Chinese FRG population [11]. The purpose of this study was to further expand the mutation analysis of SLC5A2 gene in FRG and

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investigate the relationship between genotype and RT_G. We then performed mutation gene analysis and determined the renal threshold for glucose (RT_G) in FRG patients from 9 families.

2. Materials and methods

2.1. Subjects

Twenty-one unrelated Chinese patients with FRG were included consecutively in this cohort for a study approved by the Ethics Committee of the Affiliated Hospital of Qingdao University. Informed written consent was obtained from all participants prior to participation in the study. Among the 21 cases, 14 were male and 7 were female patients. Median age at diagnosis of FRG was 42years old (range 18 years to 65 years). A total of 25 family members, including the first-degree relatives of the proband, were also included in this study. None of the participants were an offspring of consanguineous. Serum glucose hemoglobin A1c (HbA1c) and creatinine levels of all participants were checked and confirmed to be normal. None of them showed any signs of a generalized tubular dysfunction or of any other type of renal disease.

2.2. Diagnosis of renal glucosuria

The diagnostic criteria are as follows [12]: 1) 24-hour urine glucose > 0.3 g/1.73 m^2; 2) Normal glucose metabolism; 3) No evidence of other kidney disease, such as hematuria, proteinuria, acidaminuria, or phosphaturia, and normal renal function; 4) Except for pregnant women. Mild glucosuria, 24-h urine glucose < 10.0 g/1.73 m^2; medium glucosuria, 10.0 g/1.73 m^2 ≤ 24-h urine glucose < 20.0 g/1.73 m^2; severe glucosuria ≥20.0 g/1.73 m^2.

3. Methods

3.1. Blood and urine analysis

All subjects underwent urinary analysis, biochemical examination, HbA1c, fasting and 2 h postprandial blood glucose and 24 h urine glucose quantitative test. All probands received urine amino acid (Hitachi automatic amino acid analyzer L-8900) and urine phosphate determination.

3.2. Determining RT_G

The relationship between urinary glucose excretion (UGE) and blood glucose (BG) was assumed to be an idealized threshold. RT_G was determined on the basis of previous literature reports and was calculated from the following equation using measured BG, UGE and eGFR [1,13,14].

$$Rate\ of\ UGE\ (mg/min) = \begin{cases} 0 & if\ BG \le RT_G \\ GFR\ (dL/min) \times (BG\ (mg/dL) - RT_G\ (mg/dL)) & if BG > RT_G \end{cases}$$

A mixed-meal tolerance test (MMTT) containing 700 kcal (100 g carbohydrates) was ingested at $t = 0$. BG was measured at $t = -15$, 0, 30, 60, 90, 120, 180, and 240 min. Urine was collected from t = 0 to 4 h and was used to assay for glucose concentration. Urinary glucose excretion during this period is referred as UGE_{240min}. All calculations were done using Matlab software (Matlab® (computer program), Version 7.10 Natick, Mass., USA; Mathworks, Inc., 2010).

3.3. Mutation analysis

Genomic DNA was extracted from peripheral blood cells by GenElute blood genomic DNA kit (Sigma, NA2010). Mutation analysis was performed in all patients using the previous reported method of our group [11]. Amino acid substitutions previously unreported were evaluated using the *in silico* prediction programs SIFT, PolyPhen-2 and Mutation Taster. To analyze the potential effect of variants in the splice prediction, *in silico* analyses by BDGP (available at http://www.fruitfly.org) and NetGene2 (available at http://www.cbs.dtu.dk/services/NetGene2/) programs were performed. In addition, for amino acid substitutions, multiple sequence alignments with programs Chromas version 2.31 and Vector NTI Advance version 10 using SGLT2 orthologs of human (*Homo sapiens*, NP_003032.1), chimpanzee (*Pan troglodytes*, XP_009428973.2), macaque (Macaca malatta, XP_001113206.1), cattle (*Bos Taurus*, NP_976236.1), Norway rat (*Rattus norvegicus*, NP_072112.2), house mouse (*Mus musculus*, NP_573517.1), zebrafish (*Danio rerio*, NP_998091.1) and frog (Xenopus tropicalis, XP_002940641.2) also were used to evaluate evolutionary conservation.

3.4. Statistical analysis

All of the statistical analyses were performed with SPSS version 19. Statistical differences between heterozygotes and compound heterozygotes were analyzed by the Student's *t*-test. All results were presented as mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant.

4. Results

4.1. Patients and phenotype

The clinical characterization and laboratory findings of nine probands are shown in Table 1. Among of them, two patients presented with mild glycosuria (Quantitative test for 24-hour urine glucose: 1.5 and 6.8 g/1.73 m^2, respectively), four patients manifested middle degree glycosuria (Quantitative test for 24-hour urine glucose: 10.9–17.9 g/1.73 m^2), and the remaining three patients showed severe glycosuria (Quantitative test for 24-hour urine glucose: 20.2–22.7 g/1.73 m^2). Apart from these probands, all the other patients manifested mild glycosuria, and had no obvious discomfort. The 24-hour urine glucose levels of all patients and their family members are shown in Table 2.

4.2. Mutation analysis in patients with renal glucosuria

By direct sequencing analysis, twelve mutations in SLC5A2 were identified in nine families with FRG, including eleven missense variants (c.331T > C, p.W111R; c.374T > C, p.M125T; c.394C > T, p.R132C; c.601G > A, p.D201N; c.612G > C, p.Q204H; c.829C > T, p.P277S; c.880G > A, p.D294N; c.1129G > A, p.G377S; c.1194C > A, p.F398L; c.1540C > T, p.P514S and c.1573C > T, p.H525Y) and a 22-bp deletion in intron 7 (c.886(-10_-31)delGCAAGCGGGCAGCTGAACGCCC) (Table 2). All of these mutations except for c.601G > A and c.886(-10_-31)del are novel. Direct sequencing analysis did not find mutations mentioned above in 100 unrelated healthy subjects.

The data from *in silico* analysis by three different software programs SIFT, PolyPhen-2 and Mutation Taster showed that the mutations p.W111R, p.D201N, p.M125T, p.R132C, p.Q204H, p.P277S, p.G377S and p.H525Y were deleterious, and might be involved in FRG (Table 3). Three missense mutations (p.D294N, p.F398 L and p.P514S) were predicted by both PolyPhen-2 and Mutation Taster to be deleterious, however they may be “benign” in SIFT software program. The variant C.1129 G > A was located in the last base of exon 9 and adjacent to the splicing point, but it was predicted to have less effect on splicing by both BDGP and NETGENE2 software. All missense mutations are highly conserved among 8 different species (human, chimpanzee, macaque, cattle, Norway rat, house mouse, zebrafish and frog). The mutation c.886(-10_-31)del is specific in the Chinese population found by our group. In the previous study of our group, it accounted for about 37.5%

Table 1
Clinical characteristics of nine probands with familial renal glucosuria.

Clinical characteristics	Ia	IIa	IIIa	IVa	Va	*VIa*	VIIa	VIIIa	IXa
Age (years)	33	18	39	22	20	42	35	31	32
Gender	Male	Male	Male	Female	Male	Male	Male	Male	Male
Height (cm)	170	172	176	162	179	172	174	171	167
Weight (Kg)	52	66	57	47	74	76	63	64	59
BMI	18.0	22.3	18.4	17.9	23.0	25.6	20.8	21.8	21.2
FPG (mmol/L)[a]	4.1	4.3	4.0	4.2	5.3	5.7	4.4	3.9	4.4
HbA1c (%)	4.9	4.8	4.6	4.2	5.4	6.0	4.9	4.7	5.1
SCr (μmol/L)	67	89	62	71	84	88	83	75	62
eGFR (mL/min/1.73m^2)[b]	120	108	119	105	115	93	105	116	122
24 h Urine Glucose (g/24 h/1.73m^2)	13.4	17.9	25.7	20.6	6.8	1.5	12.6	10.9	20.2
Urine pH	6.0	7.0	6.0	5.5	5.5	6.0	6.0	6.0	6.0
Proteinuria	–	–	–	–	–	–	–	–	–
Aminoaciduria	–	–	–	–	–	–	–	–	–
Phosphuria	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
RT_G (mmol/L)	1.5	1.1	0.9	1.0	4.5	5.7	1.6	1.5	1.3

[a] FPG, fasting plasma glucose.
[b] 2hPBG, 2-h postprandial blood glucose; "-", negative.

(6/16) of the total alleles [11]. Of note, in this study, the deletion c.886(-10_-31)del was found in 5 out of the nine families, with a mutation rate of 28% (5/18). By calculation, the total frequency of c.886(-10_-31)del was 32% (11/34) in two studies.

4.3. Correlation of genotype and RT_G

As shown in Table 2 and Fig. 1, the compound heterozygotes cases with two mutations in SLC5A2 gene presented with much lower RT_G (1.28 ± 0.10 mmol/L) as opposed to the carriers of heterozygous variants (5.14 ± 0.77 mmol/L) ($p < 0.01$). The variant c.886(-10_-31)del heterozygotes had significant lower RT_G values than others (4.43 ± 0.37 *vs* 5.7 ± 0.51 mmol/L; $p < 0.01$).

5. Discussion

In normoglycemic subjects, the kidneys reabsorb almost 180 g of glucose each day *via* sodium/glucose co-transporter or symporters (SGLTs) to keep glucose homeostasis. The relationship between blood glucose and renal glucose filtration, reabsorption and excretion is described as a threshold type. The amount of glucose filtered increases linearly with rising blood glucose concentration and GFR; renal glucose reabsorption rises in a linear manner until its maximum (T_mG) at a splayed plasma glucose threshold, after that excretion linearly starts to increase [15]. RT_G is defined as the plasma glucose concentration at which glucose first appears in the urine. Stepped hyperglycemic clamp procedures (SHCP) has been used to determinate RT_G and T_mG both in healthy and T2DM individuals [13,16]. RT_G values were 9.5 ± 3.7 mmol/L by this means in healthy subjects. A new method, MMTT, for calculating RT_G from measurements obtained under standard clinical trial conditions has been validated by comparing with the gold-standard SHCP in healthy and T2DM subjects [13]. To our knowledge, Aires I et al. applied the approach of MMTT to estimating RT_G among FRG patients for the first time [1].

Two main forms of SGLT, SGLT1 and SGLT2, play a pivotal role in the apical membrane of proximal tubular cells in the kidneys. SGLT2

Table 2
Results of glucose excretion studies and mutation analysis in patients with familial renal glucosuria and their family members.

Family-member	24 h Glucose excretion (g/24 h/1.73m^2)	RT_G (mmol/L)	Allele1	Allele2
Ia (proband)	**13.4**	**1.5**	**p.D201N**	**p.G377S**
Ib (father)	**0.5**	**5.9**	**p.D201N**	**WT**
Ic (mother)	**1.2**	**5.3**	**WT**	**p.G377S**
IIa (proband)	**17.9**	**1.1**	**p.M125T**	**c.886(-10_-31)del**
IIb (father)	**1.9**	**4.2**	**c.886(-10_-31)del**	**WT**
IIc (mother)	**0.3**	**5.5**	**WT**	**p.M125T**
IIIa (proband)	**22.7**	**0.9**	**p.R132C**	**c.886(-10_-31)del**
IIIb (father)	**2.0**	**4.5**	**c.886(-10_-31)del**	**WT**
IIIc (mother)	**1.6**	**4.7**	**WT**	**p.R132C**
IVa (proband)	**20.6**	**1.0**	**c.886(-10_-31)del**	**p.Q204H**
IVb (father)	**0.5**	**5.6**	**WT**	**p.Q204H**
IVc (mother)	**1.2**	**3.8**	**c.886(-10_-31)del**	**WT**
Va (proband)	**6.8**	**4.5**	**c.886(-10_-31)del**	**WT**
Vb (father)	0.0	NA	WT	WT
Vc (mother)	**1.7**	**4.6**	**c.886(-10_-31)del**	**WT**
***VIa* (proband)**	**1.5**	**5.7**	**p.P277S**	**WT**
VIb (father)	**2.4**	**6.3**	**p.P277S**	**WT**
VIIa (proband)	**12.6**	**1.6**	**c.886(-10_-31)del**	**p.D294N**
VIIb (father)	**1.4**	**5.0**	**c.886(-10_-31)del**	**WT**
VIIc (mother)	0.2	NA	WT	p.D294N
VIIIa (proband)	**10.9**	**1.5**	**p.F398L**	**p.H525Y**
VIIIb (father)	0.2	NA	p.F398L	WT
VIIIc (mother)	1.0	6.4	p.H525Y	WT
IXa (proband)	**20.2**	**1.3**	**p.T111A**	**p.P514S**
IXb (son)	0.1	NA	WT	p.P514S

Patients with familial renal glucosuria are indicated by boldface; wt, wild type; na: not avaiable.

Table 3
Eleven missense variants in SLC5A2 gene and analysis results of three different online software in this study.

	Exon	Nucleotide changes	Amino acid changes	SIFT	PolyPhen-2	Mutation taster
1	Exon 4	c.331T > C	p.W111R	Damaging	Probably damaging	Disease causing
2	Exon 4	c.374T > C	p.M125 T	Damaging	Probably damaging	Disease causing
3	Exon 4	c.394C > T	p.R132C	Damaging	Probably damaging	Disease causing
4	Exon 6	c.601G > A	p.D201N	Damaging	Probably damaging	Disease causing
5	Exon 6	c.612G > C	p.Q204H	Damaging	Probably damaging	Disease causing
6	Exon 7	c.829C > T	p.P277S	Damaging	Probably damaging	Disease causing
7	Exon 7	c.880G > A	p.D294N	Tolerated	Probably damaging	Disease causing
8	Exon 9	c.1129 G > A	p.G377S	Damaging	Probably damaging	Disease causing
9	Exon 10	c.1194C > A	p.F398 L	Tolerated	Probably damaging	Disease causing
10	Exon 12	c.1540C > T	p.P514S	Tolerated	Probably damaging	Disease causing
11	Exon 12	c.1573C > T	p.H525Y	Damaging	Probably damaging	Disease causing

reabsorbs approximately 90% of the filtered renal glucose, whereas SGLT1 reabsorbs the remaining 10% [17,18]. Mutations of SGLT1 lead to glucose-galactose malabsorption, which causes potentially fatal diarrhea [19]. On the other hand, the SGLT2 mutation is the only pathogenic mechanism of FGR [11]. FRG is considered as a benign condition in general, apart from polyuria, increased frequency of urinary tract infections and activation of the renin-angiotensin aldosterone system [20]. Therefore, exploring mutation sites in FRG patients may provide a more precise target for the development of SGLT2 inhibitors.

In this study, we found twelve novel variants, including ten missense SLC5A2 mutations in Chinese FRG families. The c.374T > C, c.394C > T, c.1129 G > A and c.1540C > T missense mutations occur in 3, 3, 2 and 13 persons with a heterozygous mutation in the ExAC database, respectively. The mutation frequencies are 2.475e-05, 1.65e-05, 2.183e-05, and 0.0001, respectively. Only one person carries the c.1540C > T missense mutation in the 1000G database, and the allele mutation frequency is 0.0002. Other mutations were not recorded in ExAC and 1000G data. Therefore, based on their extremely low mutation frequency, highly conservative and predictions from biological software, it can be speculated that these missense mutations are likely to be pathogenic mutations. This study found that the c.886 (-10_-31) del mutation frequency was as high as 28%. According to our previous research data, the overall mutation frequency was as high as 32%, which was confirmed to be a high-frequency mutation again. This mutation is likely to be a hot spot mutation unique to Chinese population, which provides important reference and convenience for later genetic counseling and genetic diagnosis. Additionally, except for the high-frequency mutation c.886(-10_-31)del, most of the other mutations are still private and exclusive to a certain FRG family, which is consistent with previous studies [21,22].

RT_G is directly related to the genotype of patients with FRG, whereas 24 h urine glucose is affected by many factors such as blood glucose level and glomerular filtration rate. Therefore, the determination of RT_G is more helpful to study the relationship between genotype and phenotype in FRG patients. In the present study, our data indicated that patients with compound heterozygous mutations had significantly lower RT_G levels than patients with heterozygous mutations. This result showed that the inheritance of FRG was best described as codominant. In addition, we found that c.886(-10_-31)del carriers showed lower RT_G than other mutations carriers (4.43 ± 0.37 *vs* 5.7 ± 0.51 mmol/L; $p < 0.01$), which suggested that c.886(−10_-31)del had more severe genotype. In fact, our previous research had confirmed that this mutation caused the complete skip of exon 8 (136 bp) of the SLC5A2 gene. The c.886(-10_-31)del mutation will produce a severely truncated SGLT2, resulting in a loss of transport activity [12]. In family V, both Va and Vc were c.886(-10_-31)del heterozygous mutation, but 24 h urine glucose was quite different. This difference may relate to the effects of the mutant genes and the modified genes, diet and different levels of blood glucose metabolism cannot be ignored.

It was worth mentioned that we found that some mild FRG patients had a higher RT_G (5.0–6.4 mmol/L), and it is possible that some of these patients may show negative urine glucose under fasting state.

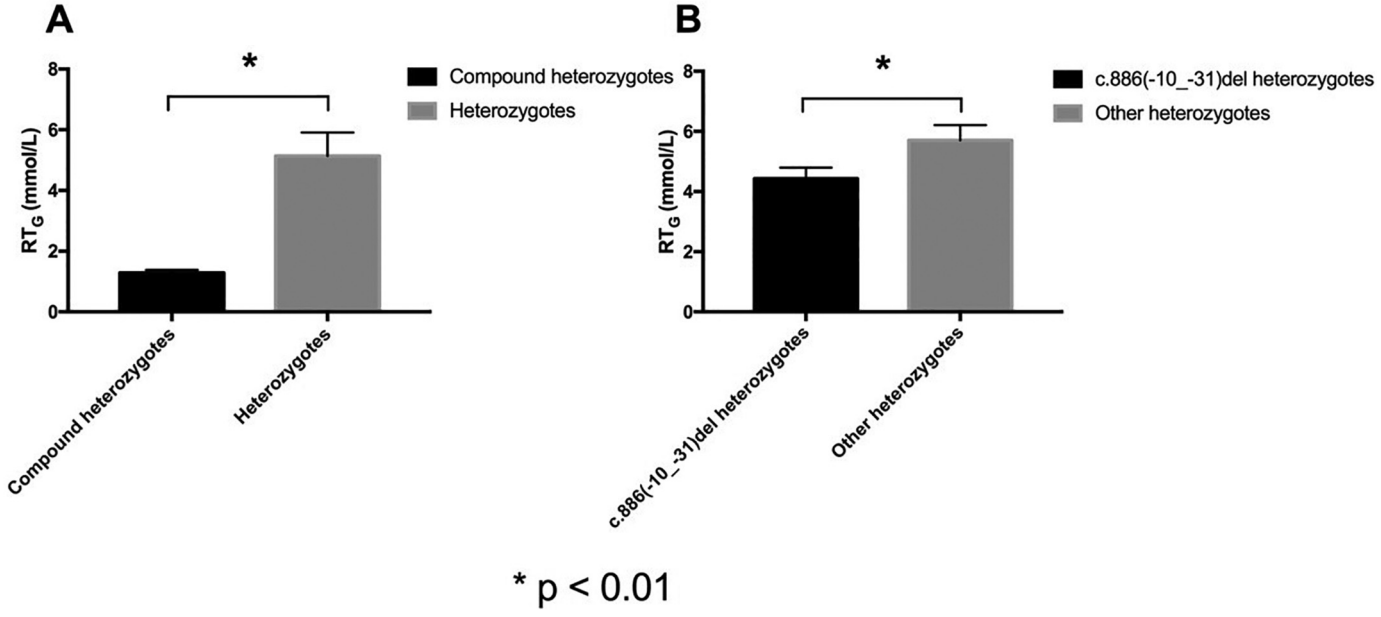

Fig. 1. Comparison of RT_G between different groups.

Therefore, enhancing postprandial urine glucose testing and expanding gene diagnosis can improve the diagnostic rate of FRG and avoid misdiagnosis of patients with mild FRG.

In summary, this study found ten novel mutations of SLC5A2 gene may be lead to FRG, and confirmed that c.886(-10_-31)del is a high-frequency mutation unique to Chinese population again. MMTT is a simple and feasible method for determining RT_G in patients with FRG. The determination of RT_G helps to establish a correlation between genotype and phenotype.

Declaration of interest

The authors declare that they have no competing interests.

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