D28G Mutation in Congenital Glucose-Galactose Malabsorption

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Background: Congenital glucose-galactose malabsorption is a rare autosomal recessive disorder of the intestinal transport of glucose and galactose, leading to watery diarrhea, dehydration, failure to thrive, and early death.

Methods: In this study, we analyzed D28G mutation in 16 family members of a patient with typical presentation of congenital glucose-galactose malabsorption with polymerase chain reaction-Restriction Fragment Length Polymorphism method.

Results: Nine members of this family were heterozygous for D28G mutation.

Conclusion: To the best of our knowledge this is the first report of D28G mutation in Iran. Moreover, this simple typical PCR-Restriction Fragment Length Polymorphism method, allows immediate identification of D28G mutation.

Keywords: Congenital glucose-galactose malabsorption (CGGM) • D28G • PCR-RFLP

Congenital glucose-galactose malabsorption (CGGM) is a rare autosomal recessive disorder, which presents as a protracted diarrhea in early neonatal life. It is due to a defect in sodium-coupled transport of glucose and galactose in the enterocyte.1 Diarrhea in CGGM is of osmotic type, caused by accumulation of unabsorbed glucose and galactose in the intestine,2 which results in severe malnutrition.3 When glucose and galactose are eliminated from the diet, infants with CGGM thrive and dietary intervention after early diagnosis will result in normal growth and development. The disease was first reported in 1962 by Lindquist and Meeuwisse from Sweden4 and Laplane et al from France.5 Since the gene that encodes the intestinal sodium-dependent glucose transporter (SGLT1) has been characterized, diagnosis of CGGM is now possible by mutation analysis.6 Wright et al have identified 46 mutations. There are 34 missense mutations. All of the nonsense, frame-shift, and splice-site mutations are predicted to produce truncated proteins, and they have demonstrated that none of the shortest and longest proteins (premature stops at codons 191 and 379) transport glucose.7

Although direct sequencing is the more accurate technique for the detection of nucleotide mutations, DNA sequencing cannot be used as a routine assay for diagnosis because sequencing protocols usually are expensive and time-consuming. Therefore, alternative protocols for the detection of single-nucleotide changes of genes have been developed that can be applied to the diagnosis in a routine clinical laboratory or epidemiological studies. For these reasons, it is of interest to us to develop a polymerase chain reaction (PCR)-based restriction fragment length polymorphism (PCR-RFLP) assay allowing rapid and reproducible identification of mutations as in previous articles.8–11 This process involves the introduction of an artificial restriction enzyme cleavage site into the PCR product using a primer-specified restriction site modification method and

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Previously, we could identify D28G mutation in the first exon of an Iranian patient by sequencing exons of SGLT1 gene. According to the high prevalence of consanguinous marriages in Iran, determination of this mutation is important for prenatal diagnosis of this lethal disease. In this study, using a PCR-RFLP method, we tried to identify D28G mutation in 16 family members of that patient.

Materials and Methods

This study was approved by the Ethical Committee of Mashhad University of Medical Sciences and was conducted at Ghaem Medical Center and Bu-Ali Research Institute, Mashhad, Iran.

Study population

Patient's background

The previously-mentioned patient, a girl with the D28G mutation, was the second child of healthy consanguinous parents. Soon after the start of breast-feeding, she developed watery stools. She was admitted with diarrhea, poor feeding, dehydration, and mild fever. She had hypernatremic dehydration, intermittent glucosuria, metabolic acidosis, and nephrolithiasis. With increasing oral feeding and decreasing intravenous fluids, diarrhea got worse and her condition deteriorated. Termination of breast-feeding and introduction of a lactose-free formula did not result in any clinical improvements. Therefore, the child’s feed was switched to a carbohydrate-free diet. Because this diet leads to cessation of diarrhea and improvement of bloating, a fructose-based formula (Galactomin 19, Nutricia) was started, after which a dramatic resolution of diarrhea occurred. Her biochemical tests corrected soon, and her weight increased rapidly. In the last follow-up visit, she had normal development and growth.

Family members

This study was performed on 14 family members of a girl with classic presentation of CGGM who had D28G mutation. We depicted her first and second-degree pedigree. After receiving an informed consent, the patient, her parents, and 14 persons in the pedigree were included in this study (Figure 1). The persons I-4, II-8, and II-13 had been died at the time of study. We could not receive consent for persons II-10 and III-1. We have done PCR-RFLP for 16 persons of this family.

Healthy persons

We tested the validity of our method using genomic DNAs of 20 healthy subjects out of this family. They were healthy persons with normal history and physical examination for gastro-intestinal problems.

Molecular methods

Our strategy was to amplify the first exon of SGLT1 gene using specific primers. This product could be digested by EcoRV enzyme, if the D28G mutation was absent.

Genomic DNA was isolated from whole blood collected with EDTA as anticoagulant, using the “salting out” method with commercial Biogene kit (Mashhad, Iran). In this method, after RBC lyses and WBC precipitation with reagents in different salt concentrations, protein is removed. Then, DNA is extracted by ethanol and desalted. Finally soluble DNA is stored at -20°C.

A specific portion of SGLT1 gene (first exon) was amplified by PCR. The PCR was performed in 20 µL reaction, containing 100 – 150 ng DNA, 500 µM of specific primers, 0.5 U Taq DNA polymerase (CinnaGen), 1× PCR reaction buffer (10 mM/L Tris-HCl, 50 mM/L KCl, 1.5 mM/L MgCl2), 0.2 mM each dNTP, 0.5µM each primer, 10× PCR buffer, 200 µM dNTP’s, and 1.5 mM MgCl2. The PCR protocol was five minutes at 94°C for the first cycle; then one minute at 94°C; one min at 62°C; one min at 72°C for 35 cycles, and an elongation time of five min at 72°C was used at the last cycle. Corbett thermocycler (Corbet Research, Australia) was used. The template for amplification was exon 1 of SGLT1 gene. The primers were as follows: sense; 5’-CAT TCG CAG GAC AGC TCT TA-3’ and antisense; 5’-TAA AGT GCT TCC AAG TCA TT-3’ (exon 1).

Thereby, a 521-bp fragment length was amplified. PCR products were digested by 1 U of restriction enzyme EcoRV (Fermentas, Germany, 10 U/µL) for 4 µL PCR product and incubated overnight at 37°C. The cleaved DNA fragments were subjected to electrophoresis on 2% agarose gel. We have also sent the PCR product of the proband patient for sequencing.

Results

Using the above-mentioned method, we were
able to develop a new PCR-RFLP test for the detection of D28G mutation in the SGLT1 gene.

The restriction enzyme EcoRV used in this PCR-RFLP procedure, cuts 5′GAT-ATC3′. The mutation was D28G (D=Asp=GAT, G=Gly=GGT). Figure 2 shows the partial sequence of the normal and mutant SGLT1 gene around the mutation site.

For normal DNA samples, there was complete cleavage of the 521-bp DNA fragment as expected (Figure 2: lane 5). Two shorter fragments (190 and 331 bp) were produced from a homozygous non-mutant DNA (Figure 2: lanes 3 and 4). Heterozygous DNA samples from persons in the pedigree (Figure 2: lanes 1 and 2) yielded three fragments (521, 190, and 331 bp, respectively).

According to the pedigree, the parents of the proband were relatives (Figure 1). It seems that the mutated gene has been inherited from maternal grandmother and paternal grandfather in this pedigree. The results of PCR-RFLP analysis of proband patient were in agreement with the results from DNA sequencing. In this family, persons I-2, I-3, II-2, II-4, II-6, II-7, II-9, II-11, and II-15 were heterozygote for D28G mutation.

There was a history of diarrhea and failure to thrive (FTT) in infancy in six out of nine heterozygotes. None of the seven normal homozygotes had history of FTT and infantile diarrhea. There was a correlation between the presence of one D28G mutation and clinical presentation in infancy (P<0.0001).

**Discussion**

To the best of our knowledge, this is the first report of D28G mutation analysis in Iran. The method used in this study requires only PCR and one enzyme, and is technically less demanding than most other molecular biological approaches.

PCR-RFLP is an easy and rapid method of mutation detection that is very useful for screening of family of affected patients.

SGLT1, an isoform of Na⁺-dependent glucose-galactose malabsorption
co-transporters, is localized at the apical plasma membrane in the epithelial cells of the small intestine and the proximal renal tubule, where they play a central role in the absorption of glucose and galactose from food and the reabsorption of glucose from the glomerular filtrate.13

The products of D28G clones are localized in the cytoplasm, showing that the aspartic acid-28 may be essential for the delivery of SGLT1 to the plasma membrane. This suggests that a short amino acid sequence of the N-terminal domain of SGLT1 plays important roles in plasma membrane targeting and specific apical localization of the protein.13

CGGM is characterized by a neonatal onset of severe diarrhea.7 The diarrhea stops immediately after removing the offending sugars (glucose, galactose, and lactose) from the diet, but promptly resumes on feeding. The location of SGLT1 was refined to 22q13.1. Homozygous mutations are observed in 60%, in agreement with the autosomal recessive nature of the disease.14

Wright et al have identified homozygous mutations in 30 CGGM patients, and heterozygous mutations in 16 CGGM patients (in 12 of the latter cases, they have identified the mutations on both alleles). Their DNA analysis of CGGM patients and their near relatives has confirmed the autosomal recessive nature of the disease.7

In the present study, patient had D28G mutation in SGLT1 protein. In this mutation, aspartic acid (GAT) is substituted by glycine (GGT) due to a point mutation (A→G) in the genome. The previous results of molecular analysis showed that our patient had D28G mutation.

Although demonstrating a co-transporter defect after small intestinal biopsy makes a definitive diagnosis of CGGM, dietary manipulation may suffice in establishing it.15 In this patient, the diagnosis of CGGM seems certain based on the clinical presentation and the response to dietary manipulations and beginning of diarrhea when specific formula discontinued.

Although various DNA-based molecular methods have been described, a simple, reliable, and cost-effective method is needed for mutation analysis. Accurate and reproducible methods are essential for epidemiological purposes. Various molecular methods have been used. However, the sequencing method is both time-consuming and labor-intensive and therefore cannot be used as a rapid diagnostic method.

The D28G mutation in the SGLT1 gene does not create or destroy a restriction endonuclease site, and hence a direct PCR-RFLP method cannot be used for its detection. Due to sequence complexity around the D28G mutation site, we...
designed an indirect method by using EcoRV enzyme for digestion of nonmutant sequence. In addition, there were some nonspecific PCR products. We tried various PCR conditions including different annealing temperatures in the range of 55 to 62°C that resulted in good yields (data not shown). Subsequent treatment of this fragment with EcoRV allowed us to detect the D28G mutation either in its homozygous or heterozygous state.

Surprisingly, none of the samples from healthy persons of this family contained the D28G mutation in the homozygous or heterozygous form. The detection of D28G mutation by PCR-RFLP method described in this paper could be a valuable complement to clinical screening methods in identifying neonates with congenital diarrhea. Early diagnosis of such infants becomes particularly important for treatment and management. DNA-based detection of D28G mutation in the SGLT1 gene would also be useful to determine the prevalence of carriers in the general population to provide a better genetic counseling in future. Studies with a larger group of patients should reveal the exact status of the D28G mutation in Khorasan Province, northeastern Iran.

This simple typical PCR-RFLP method was applied to amplicons from the first exon, allowing immediate identification of D28G mutation. The results of PCR-RFLP analysis were comparable with those of sequencing of the region. According to this study, one can conclude that D28G mutation may have some presentation in heterozygous patients.

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