MUTATION IN BRIEF

Mutation Analysis of the GALT Gene in Czech and Slovak Galactosemia Populations: Identification of Six Novel Mutations, Including a Stop Codon Mutation (X380R)

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A study of the galactose-1-phosphate uridyltransferase (GALT) gene from 37 unrelated galactosemia families is reported here. A total of 16 sequence variations in eleven mutated alleles was found. The two most common molecular defects were the mutations Q188R (46.0%) and K285N (25.7%). Six novel mutations in the GALT gene, X380R, Y209S, E340K, L74fsdelCT, Q169K and L256/P257delGCC, were detected. Three mutations, V151A, L195P and R204X that were previously described in other populations, were also found. The mutation X380R, which breaks the stop codon of the GALT gene, causes elongation of the GALT enzyme's protein chain. A deletion of four nucleotides in the 5' promoter region, in a position 116 - 119 nucleotides upstream from the initiate codon (5'UTR-119delGTCA), was revealed in Duarte (D2) alleles, in addition to N314D, IVS4nt-27g→c, IVS5nt+62g→a, and IVS5nt-24g→a. An unusual molecular genotype was observed on 2 types of classical galactosemia alleles, with six variations from the normal nucleotide sequence presented in cis (mutation V151A or E340K plus five Duarte (D2) characteristic variations). In summary, galactosemia is a heterogeneous disorder at the molecular level, and mutation N314D, appears to be an ancient genetic variant of the GALT gene.

KEY WORDS: galactosemia, GALT gene, mutation profile, protein elongation test

INTRODUCTION

Galactosemia (MIM# 230400) is an autosomal recessive inherited metabolic disorder caused by a defect in the galactose-1-phosphate uridyltransferase (GALT) enzyme. The absence or severe reduction of GALT activity results in classical galactosemia (G/G), while an approximately 50% reduction of enzyme activity leads to the

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Duarte variant of galactosemia (D/D). Classical galactosemia is characterized by diarrhea, vomiting, failure to thrive, jaundice, hepatosplenomegaly and cataracts. When not treated with a galactose-restricted diet, infants with galactosemia develop sepsis that leads to death.

Cloning and sequencing of GALT cDNA (Reichardt and Berg, 1988; Flach et al., 1990) and of the human GALT gene (Leslie et al., 1992) enabled investigation of defects of this gene on the DNA level. Mutation Q188R was found to be the most common molecular defect among Caucasian classical galactosemia patients (Reichardt et al., 1991; Elsas et al., 1993; Ng et al., 1994), whereas N314D was predominantly detected in Duarte galactosemia patients (Elsas et al., 1994; Lin et al., 1994). In recent studies, the Duarte (D2) allele, with 50% of normal GALT activity, and the Los Angeles (D1) allele, with 110-130% of normal GALT activity, were characterized as having nucleotide alterations in addition to N314D (Podskarbi et al., 1996; Greber-Platzer et al., 1997; Kozák et al., 1997a; Langley et al. 1997). More than 100 disease-causing mutations in the GALT gene have been identified and are cited in the GALT Mutation Database (Tyfield, 1998).

This report describes results of molecular analysis of the GALT gene in 37 unrelated Czech and Slovak families with galactosemia.

MATERIALS AND METHODS

Subjects

Thirty-nine patients and their relatives were investigated. These patients came from 37 unrelated galactosemia families from the Czech and Slovak Republics (29 Czech and 8 Slovak families). All families were Caucasians. Thirty-eight patients were classified as having classical galactosemia on the basis of residual GALT activity less than 3% of the control value (Beutler and Baluda, 1966), one patient was classified only on the basis of clinical and biochemical parameters (galactose, galactose-1-phosphate, galactitol). Genomic DNA was extracted from 10 ml of EDTA-anticoagulated blood using standard methods.

DGGE Analysis

Denaturing gradient gel electrophoresis (DGGE) was used to detect mutations in exons 5, 7 and 10 of the GALT gene, together with their flanking intronic sequences. Genomic DNA was amplified using PCR primers: 5' [GC]-TTGGAGACTCAGCATTGGGG 3' and 5' ACTCCCTCCTGACCACACCC 3' (exon 5); 5' [GC]-ATGTGGAGGCTTGGAGGTAA 3' and 5’ TTCACCTCTAGCTTTCTCCT 3' (exon 7); 5' [GC]-TTGGGTTTGGGAGTAGGTGC 3' and 5' GGGCAACAGAAGTATCAGGT 3' (exon 10) where 5‘ [GC] = CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG. Amplified fragments were run in 6% polyacrylamide/TAE gel containing a linear gradient of denaturant (100% denaturant = 9.5M urea). Generation of heteroduplexes and DGGE electrophoresis was performed under the conditions described by Kozák et al., 1997b.

Heteroduplex Analysis

Heteroduplex analysis (HA) was used as the second mutation screening method for detection of mutations or potential polymorphisms in remaining eight exons of the GALT gene. After amplification, PCR products were denatured at 100°C for seven minutes, and then held at 37°C for 30 minutes. Ten µl of PCR product was mixed with 2µl of non-denaturing loading dye (6xTriple Dye, FMC), and samples were loaded onto a 1x MDE gel (FMC) containing 2.5M urea and using 0.6x Tris-borate-EDTA (0.6xTBE) as the running buffer. Electrophoresis was run for 20 hours at 300 V in room temperature. The gels were stained with ethidium bromide.

Sequence Analysis

Sequence analysis was performed using solid-phase sequencing (Hultman et al., 1989). Strand separation of the PCR products was obtained using streptavidin-coated magnetic beads M280 (Dynal). The resulting single-stranded PCR product served as the sequencing template. Sequencing reaction and electrophoresis were carried out according to procedures described previously (Kozák et al., 1997b).

PCR/Restriction Enzyme Digestion Analysis

A rapid PCR-based DNA analysis was used to confirm or identify the sequence variations that create or abolish the given natural and amplification-created restriction sites. The PCR primers, appropriate enzymes and characterization of normal and mutant alleles are presented in Table 1. Enzymatic digestion of PCR products and analysis of obtained fragments were performed as described elsewhere (Kozák et al., 1995).

Table 1: Mutations or sequence variations, primers used for PCR and appropriate restriction enzymes used in detection of normal and mutant alleles.
(ACRS - amplification created restriction site)

MUTATION	PRIMER	Enzyme	Detection	
			Normal allele [bp]	Mutant allele [bp]
Q188R	5' TGGATGGGCAGGGAGGGGGT 3' 5' TGTCAAGGGGCAAAAGCAGA 3'	Hpa II	348	184+164
K285N	5' TGGGGCTAGGCACTGGATGGA 3' 5' AGG ACG TCT CAA AGA GGT TGT CGT A 3'	Rsa I (ACRS)	117+24	141
X380R	5' GGA GAC AGC AAC CAG CGC 3' 5' TGCTATATCTGCCCAAATTCC 3'	AlwN I (ACRS)	109+18	127
L195P	5' TCA GGG GCT CCA GTG GGT TTC 3' 5' TCA CGC TGG GCA ATA TCT GCC 3'	Hpa II (ACRS)	194	172+22
Q169K	5' ACA GCC AAG CCC TAC CTC CCG 3' 5' ACTCCCTCCTGACCACACCC 3'	Hph I	201+52+30	154+52+74+30
L256/P257delGCC	5' CGGCTCCTATGTCACCTTGAT 3' 5' CAACCTCCATCCAGTGCCTAG 3'	Bgl I	135+94	226
N314D	5' GGGTTTGGGAGTAGGTGCT 3' 5' GGGCAACAGAAGTATCAGGT 3'	Ava II	213+94	111+102+94
L218L	5' ATGTGGAGGCTTGGAGGTAAA 3' 5' TTCACCTCTAGCTTTCTCCT 3'	Mse I	272	147+125
IVS4nt-27g→c	5' ACA GCC AAG CCC TAC CTC CCG 3' 5' ACTCCCTCCTGACCACACCC 3'	Msp I (ACRS)	171+93+19	171+112
IVS5nt+62g→a	5' TGGATGGGCAGGGAGGGGGT 3' 5' TGTCAAGGGGCAAAAGCAGA 3'	Dde I	222+120+6	186+120+36+6
IVS5nt-24g→a	5' TGGATGGGCAGGGAGGGGGT 3' 5' TGTCAAGGGGCAAAAGCAGA 3'	Sac I	239+109	348
5'UTR-119delGTCA	5' CAG GGC AGC CCA GTC ACT CA 3' 5' GCGTTGCTGAGGATCGGTTC 3'	Dde I (ACRS)	145+17+10	158+10

Protein Elongation Test

Total RNA and mRNA were extracted from peripheral blood leukocytes of one X380R heterozygote subject and one control subject using RNeasy Mini Kit (Qiagen, Germany) and mRNA Isolation Kit (Boehringer Mannheim, Germany), respectively, according to manufacturer's instructions. RT-PCR was carried out using the Titan^{TM} One Tube RT-PCR System (Boehringer Mannheim, Germany) and the following primers: RT4.1A (5' CCC AGT GAT CAT CCC CTT TTC 3') and 11B (5' TGC TAT ATC TGC CCA AAT TCC 3'). One microliter of the amplified RT-PCR product was then added to a 25 µl PCR mixture containing 0.5 µM of primers RT5.2APTT (5' GGA TCC TAA TAC GAC TCA CTA TAG GAA CAG ACC ACC ATG TCG GAT GTA ACG CTG CCA CT 3') and 11B (mentioned above, located in 3'UTR region very close to the polyadenylation signal). Two microliters of unpurified nested T7-PCR product were used for in vitro transcription/translation reaction with ^{35}S-methionine (Amersham, UK), using the conditions recommended by the TnT^{TM} T7-coupled reticulocyte lysate system (Promega, Madison). An aliquot of 5 µl of the translation products was mixed with 10 µl of gel-loading buffer, heated to 100ºC for three minutes and separated (4 hrs. at 80 V) on a 15% discontinuous SDS-PAGE gel with a thickness of 0.75 mm. The gel was dried and the signal was detected by autoradiography.

RESULTS

Mutation analysis was performed for the 5' upstream region and the whole coding region with flanking intronic sequences of the GALT gene using PCR/digestion, DGGE, HA and sequencing approaches. After screening of 37 galactosemia patients (one from each family) and their relatives, six novel candidate galactosemia mutations and ten previously identified base substitutions in exon or intron sequences were found. Altogether eleven mutated alleles were found. The results are summarized in Table 2.

Table 2: Distribution and frequency of galactosemia mutations in 74 mutant alleles of the GALT gene from the Czech and Slovak Republics

Mutation		No. of alleles	Frequency (%)
Trivial Name	Systematic Name		
Q188R	c. 563 A→G	34	46.0
K285N	c. 855 G→T	19	25.7
X380R	c. 1138 T→C	4	5.4
V151A+ N314D 5'UTR-119delGTCA IVS4nt-27g→c IVS5nt+62g→a IVS5nt-24g→a	c. 452 T→C c. 940 A→G c. -119→-116delGTCA c. 378-27g→c c. 507+62g→a c. 508-24g→a	3	4.1
L195P	c. 584 T→C	3	4.1
Y209S	c. 626 A→C	2	2.7
E340K+ N314D 5'UTR-119delGTCA IVS4nt-27g→c IVS5nt+62g→a IVS5nt-24g→a	c. 1018 G→A c. 940 A→G c. -119→-116delGTCA c. 378-27g→c c. 507+62g→a c. 508-24g→a	2	2.7
L74fsdelCT	c. 220 – 221delCT	1	1.3
Q169K	c.505 C→A	1	1.3
R204X	c. 610 C→T	1	1.3
L256/P257delGCC	c. 768 – 770delGCC	1	1.3
Total		**71**	**95.9**

Underlines denote mutations not previously described

In the first step, the prevalent mutation, Q188R, was screened by PCR and restriction analysis (Table 1). Thirty-four (46.0%) Q188R mutant alleles were detected. Ten patients were homozygous and 14 were heterozygous for the Q188R mutation.

DGGE analysis of exons 5, 7 and 10 showed several abnormal migration patterns, indicating that the analyzed DNA contains various mutations or sequence alterations in these regions. Two point mutations, novel Q169K

(CAG→AAG) and previously described V151A (Elsas et al., 1995), were found subsequently by sequencing of exon 5. Besides this, a G→A nucleotide change at position 1323 in intron 5 (IVS5nt+62g→a) and a G→C substitution at position 1105 (IVS4nt-27g→c) in intron 4 were detected through sequencing. Together with the third substitution at position 1391, IVS5nt-24g→a (Lin and Reichardt, 1995) and with the GTCA tetranucleotide deletion in the 5' promoter region (5'UTR-119delGTCA) (Kozák et al., in press), they are nucleotide alterations typical for the Duarte (D2) alleles. A novel mutation Y209S was found in exon 7 on two mutant alleles in two unrelated families (2.7%). In addition, previously described mutations L195P and R204X were revealed in exon 7 on three and one mutant alleles, respectively. DGGE and sequencing analysis of exon 10 uncovered a novel mutation E340K (GAA→AAA) and the common Duarte mutation N314D. Mutations V151A and E340K were detected in patients: one V151A/V151A homozygote, one V151A heterozygote and one E340K/E340K homozygote. These patients also had N314D, 5'UTR –119delGTCA, IVS4nt-27g→c, IVS5nt+62g→a, and IVS5nt-24g→a in cis, in the homozygote and heterozygote states. The presence of mutations V151A or E340K and all Duarte (D2) alterations on the same allele (in cis) was confirmed in corresponding families by analysis of all family members.

Heteroduplex analysis (HE) was used to reveal mutations in exons 1-4, 6, 8-9 and 11 of the GALT gene. Abnormal band patterns were observed in exons 2, 8 and 9. After sequencing of exons 2 and 8, novel mutations were identified: L74fsdelCT due to deletion of CT in codon 74 and L256/P257delGCC due to deletion of GCC in codons 256 and 257. Sequence analysis of exon 9 revealed mutation K285N (AAG→AAT). Amplification-created restriction site (ACRS) approach enabled us to easily confirm the K285N mutation on 19 (25.7%) mutant alleles.

In seven patients (three Q188R/?, one K285N/?, one L195P/?, one Y209S/?, and one Q169K/?), seven mutant alleles remained unspecified after the above-mentioned analyses. For this reason, the whole coding region with flanking intronic sequences plus the 5' upstream region was sequenced for these patients. Only one other sequence alteration, a novel mutation TGA→CGA which substitutes the amino acid, arginine, for the stop codon at residue 380 (X380R), was found in four patients. To confirm that this rare type of mutation breaks the stop codon, a protein elongation test was performed. As shown in Fig. 1, the mutation X380R causes elongation of the GALT enzyme's protein chain.

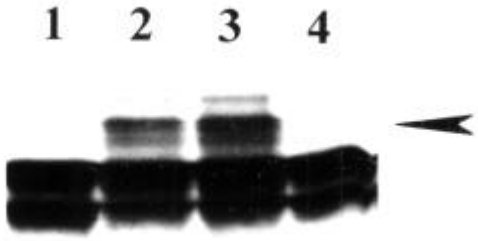

Figure 1. Protein elongation test. Protein patterns detected in SDS-PAGE gel. Lane 1: results from total RNA of a normal control; lane 2: results from total RNA of the X380R heterozygote patient; lane 3: results from mRNA of the X380R heterozygote patient; lane 4: results from mRNA of a normal control. Arrow indicates the elongated protein.

DISCUSSION

By detailed scanning of the GALT gene, a mutation detection rate of 95.9% was achieved. The most frequent galactosemia mutation, Q188R, was found in 34 (46.0%) mutant alleles. This frequency is lower than that in previous studies of Caucasian populations (from 91% down to 58%) (Leslie et al., 1992; Elsas et al., 1993; Ng et al., 1994; Podskarbi et al., 1994; Murphy et al., 1996; Greber- Platzer et al., 1997; Tyfield et al., 1997). Our results

suggest that the frequency of Q188R decreases from west to east across Europe. The second common galactosemia mutation in our patients, K285N, accounted for 25.7% of the galactosemia alleles. The K285N mutation was relatively rarely detected in recent studies. Only in the Austrian population does K285N appear with a frequency similar to that for our population (Greber-Platzer et al., 1997). As the results show, K285N is more prevalent in Central Europe. Based on preliminary results from a study done in Poland (Zekanowski, personal communication), as well as on the study presented here, we suggest that the K285N mutation probably has a Slavic origin.

Besides two common galactosemia mutations, nine other types of galactosemia alleles were detected (Table 2). One novel candidate galactosemia mutation, Y209S, was revealed in exon 7. The triplet codon 209 is in an evolutionarily conserved amino acid sequence. For these reasons, we conclude that nucleotide alterations in codon 209 severely affect enzyme activity. The next two novel mutations mentioned, L256/P257delGCC and X380R, change the protein structure of GALT by deletion of the 257th amino acid (proline) and by breaking the stop codon, respectively. A protein elongation assay done for the X380R heterozygote confirmed that the X380R mutation indeed breaks the stop codon and causes elongation of the GALT protein chain (Fig. 1). The change of protein structure probably leads either to increased degradation of the mutant enzyme in cells by proteolytic cleavage or to increased conformation (intrinsic) instability of the GALT protein. Both possibilities would lead to a decrease in enzymatic activity. In light of the fact that compound heterozygotes Q188R/X380R and L195P/X380R result in classical galactosemia, we assume that the X380R mutation severely alters GALT activity. Two other mutations, R204X and L74fsdelCT, change the protein structure of GALT by creating a premature stop codon.

When pedigree analysis was performed on affected families with more than two nucleotide changes, the original types of the mutant alleles were distinguished. The V151A and E340K mutations were in linkage together with the N314D mutation, with the 5’ promoter alteration 5'UTR–119delGTCA and with three intron sequence changes: IVS4nt-27g→c, IVS5nt+62g→a, IVS5nt-24g→a (Table 2). This fact is not too surprising - few distinct mutations were previously detected on Duarte (D2) alleles (Elsas et al., 1995; Gathof et al., 1995; Sommer et al., 1995; Podskarbi et al., 1996).

To conclude, in this study we confirm the molecular heterogeneity of classical galactosemia. Sequence alterations in 4.1% of alleles still remain unspecified. The unknown defects may possibly lie deep in the intronic regions and may exert some effect on splicing.

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