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Frequency of Galactose-1-phosphate Uridyl Transferase Gene Mutations in Healthy Population of Croatia

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• galactosemia
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• N314D
• IVS5-24 G>A

Galactosemia is a human disease caused by deficient activity of each one of the three enzymes involved in galactose metabolism, galactokinase (GALK), galactose-1-phosphate uridyl transferase (GALT) and UDP-galactose-4-epimerase (GALE). Absence or deficiency of GALT activity results in classical galactosemia. This disorder exhibits allelic heterogeneity in different populations and ethnic groups. The aim of this study was to search for galactosemia mutations Q188R, N314D, and K285N in healthy population of Croatia. DNA samples from 221 subjects were analyzed by the polymerase chain reaction, followed by digestion with restriction endonucleases (PCR-RFLP procedure). Allele frequencies for Q188R, N314D, and K285N were found to be 0.2 %, 7.5 % and 0 %, respectively, and correlate well with those published for most other healthy Caucasian populations.

INTRODUCTION

Galactose is metabolized in three steps, which are catalyzed by galactokinase (GALK), galactose-1-phosphate uridyl transferase (GALT) and UDP-galactose-4-epimerase (GALE).[1] Galactose enters energy metabolism due to its conversion into glucose-1-phosphate by GALT or is incorporated into glycoproteins and glycolipids as UDP-galactose, another product of GALT reaction, which is the substrate of all galactosylation reactions.[2]

Galactosemia is a human disease caused by deficient activity of each one of the three enzymes involved in galactose metabolism. Galactosemia is characterized by a variety of symptoms such as cataracts, failure to thrive, vomiting, diarrhoea, hepatomegalia, sepsis, ovarian failure, which if not treated may lead to death.[1]

Absence or deficiency of GALT activity results in classical galactosemia (McKusick 230400).

The human GALT gene is mapped to chromosome 9p13.[3] The gene containing 11 exons and spanning 4 kb has been cloned and sequenced. More than 50 point mutations have been identified by the single-strand conformation polymorphism (SSCP) analysis.[4] The most common classical galactosemia mutation is substitution of glutamine at codon 188 with arginine (Q188R).[4–6] This mutation prevails in Caucasian population and has been reported to account for 54 to 70 % of classical galactosemia alleles. Homoallelic patients with Q188R mutation exhibit a complete loss of GALT enzyme activity, but heterozygotes have approximately 50 % of normal GALT activity. The S135L mutation is the most frequently observed

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TABLE I. Frequencies of Q188R, N314D, IVS-24G>A, Duarte- 2, Duarte-1 and K285N mutations in healthy population of Croatia

	Q188R	N314D (D1 + D2)	IVS5-24G>A	Duarte-2	Duarte-1	K285N
Number of cases	221	221	221	221	221	221
Number of heterozygotes	1	31	19	16	15	0
Number of homozygotes	0	1	0	0	1	0
Number of all alleles	442	442	442	442	442	442
Number of normal alleles	441	409	423	426	425	442
Number of mutated alleles	1	33	19	16	17	0
Frequency of normal alleles / %	99.8	92.5	95.7	96.4	96.2	100.0
Frequency of mutated alleles / %	0.2	7.5	4.3	3.6	3.8	0.0

mutation in African-Americans and accounts for approximately 50 % of the mutant alleles in that population. The K285N mutation is common in individuals of Eastern European descent and accounts for 8 % of alleles in the general European population. Homoallelic patients with this mutation exhibit a complete loss of GALT enzyme activity, but heterozygotes have approximately 50 % of normal GALT activity.[7] Another two GALT variants are named after two cities, Duarte (D2 or Duarte-2) and Los Angeles (D1, Duarte-1 or Los Angeles, LA) where they were first observed. These variants are caused by the substitution of asparagine to aspartate at position 314 (N314D). Both variants are associated with changed electrophoretic mobility of GALT due to increased overall negative charge of mutant protein, but very different enzyme activities. D1 is associated with increased GALT activity (110–130 % of normal activity), and D2 with reduced activity (about 50 % of normal activity), depending on additional regulatory genetic changes in the alleles.[8,9] It has been reported that D1 allele also carries the L218L silent mutation in exon 7, *cis* with N314D, and D2 alleles, besides N314D mutation, carry three intronic sequence variations (IVS), IVS4-27G>C (G1105C), IVS5+62G>A (G1323A) and IVS5-24G>A (G1391A) in intron 5 together with 5' promoter deletion – 119_116 del-GTCA.[8,10] The D2 variant is present in 5 % of the general U.S. population.[11,12] Duarte, N314D mutation, when paired with a classical galactosemia mutation, results in a milder and probably benign phenotype with approximately 75 % reduction in enzyme activity.

Although heterozygotes for classical and Duarte galactosemia appear to be benign, there are some indications that these disorders increase the risk of developing ovarian failure and presenile cataracts later in life.[13,14]

The aim of the presented study was to analyze healthy Croatian population for the frequency of galactosemia alleles Q188R, N314D, K285N, and the intronic variant IVS5-24G>A. The results of this study provide a basis for examining groups of patients with diseases associated with each mutation.

EXPERIMENTAL

Subjects

Two hundred and twenty-one healthy subjects from eastern, southern and central parts of Croatia were screened for frequencies of Q188R, N314D, K285N mutations and the IVS5-24G>A intron variation. The study was approved by the Ethics Committees of the Clinical Hospital Split, »Dr. Josip Benčević« General Hospital, Slavonski Brod, and Clinical Hospital for Lung Diseases, Jordanovac, Zagreb.

Samples

Genomic DNA was extracted from 2.5 ml EDTA blood by the salting out procedure.[15]

Polymerase Chain Reaction (PCR)

PCR was performed for four DNA fragments containing potential sites of intron variation (IVS5-24G>A, intron 5) and Q188R (exon 6), N314D (exon 10), and K285N (exon 9) mutations. Each PCR mixture (total volume 25 μl) contained 250 ng genomic DNA, 0.4 μmol dm^{-3} of each primer (Microsynth GmbH), 0.2 mmol dm^{-3} dNTP (mixture of dATP, dGTP, dCTP, dTTP; 0.2 mmol dm^{-3} of each; Pharmacy Biotech), 2 mmol dm^{-3} $MgCl_2$ (Invitrogen), PCR buffer (20 mmol dm^{-3} Tris-HCl pH = 8.4, 50 mmol dm^{-3} KCl; Invitrogen) and 0.5 units of Platinum *Taq* polymerase (1 unit of Platinum *Taq* DNA Polymerase is the amount of enzyme required to incorporate 10 nmol of deoxyribonucleotide into acid-precipitable material in 30 minutes at 74 °C; Invitrogen). The following primers were used in PCRs to amplify:

a) 572 base pairs (bp) fragment containing IVS5-24G>A intron variant and Q188R mutation,
F: 5'-AACCAGAGTTGGAGACTCAGCA-3',
R: 5'-AGAACAGGCAGGTCCTTTACCT-3';
b) 450 bp fragment containing N314D mutation,
F: 5'-CCTAGTGAACTGCAACCTCAAA-3',
R: 5'-GAGACGCCAGACTGTTCTGAGT-3';
c) 715 bp fragment containing K285N mutation,
F: 5'-GATGGAGGTTGCTCCCAGTA-3',
R: 5'-AGCACAAGGGCAACAGAAGT-3'.[16,17]

PCR for all fragments consisted of one cycle of 12 min at 95 °C, 35 cycles with three steps (first at 94 °C for 30 s, second at 55 °C for 30 s and third at 72 °C for 60 s) and one cycle of 7 min at 72 °C. PCR was attenuated by lowering the temperature to 4 °C for at least 6 min.

Detection of Q188R, N314D, K285N mutations and the IVS5-24G>A Intron Variant by Restriction Fragment Length Polymorphism (RFLP)

Amplified DNA fragments were searched for the Q188R mutation based on an additional restriction site for endonuclease *Hpa*II, which is created by this mutation in exon 6 of the GALT gene. Likewise, N314D and K285N create additional restriction sites for *Ava*II in exon 10 and for *Tsp*509I in exon 9 of the GALT gene. Intron variation, IVS5-24G>A, eliminates the *Sac*I restriction site present in a normal GALT gene.

Each restriction endonuclease mixture (total volume 15 μl) contained 5 ml of amplified fragment, appropriate buffer for each restriction endonuclease (New England Biolabs) and 2.5 units of each restriction endonuclease (1 unit of restriction enzyme is the amount of enzyme required to digest 1 μg of lambda DNA in 1 hour at appropriate temperature; New England Biolabs). Restriction products were separated by electrophoresis (100 V, 60 mA, for 60 min) on 4 % agarose gel (NuSieve® 3:1 Agarose, Cambrex Bio Science Rockland, Inc.) in TAE buffer (0.04 mol dm^{-3} Tris-HCl pH = 7.9, 5 mmol dm^{-3} Na-acetate, 0.04 mmol dm^{-3} EDTA). Gels were stained with ethidium bromide (0.5 μg/ml).

Statistical Analysis

Comparisons of the frequencies of Q188R and K285N mutations were analyzed by the Z-test. The chi-square test was used for comparisons of the frequencies of N314D (Duarte-1 and Duarte-2) mutation.

RESULTS AND DISCUSSION

In the present study, a healthy population of two hundred and twenty-one healthy volunteers from different regions of Croatia was searched for Q188R, N314D, K285N and IVS5-24G>A mutations in the GALT gene. All the mentioned mutations were estimated by the PCR-RFLP procedure.

Frequency of Q188R Mutation

In order to estimate the Q188R mutation in exon 6 of the GALT gene, the 572 bp fragment was amplified by PCR using forward and reverse primers as mentioned in Experimental. Q188R mutation introduces an additional restriction site for *Hpa*II endonuclease, giving specific restriction fragment patterns after digestion of the amplified fragment with *Hpa*II. If one allele is mutated (heterozygote, carrier of classical galactosemia allele), restriction with *Hpa*II generates four fragments of 415, 273, 157 and 142 and, in the case of a normal allele, two fragments of 415 and 157 bp. Figure 1a shows the characteristic restriction pattern for heterozygote (lane 1) and normal alleles (lanes 3, 4, 5). In fact, in the case of heterozygote, only three fragments could be detected on stained agarose gel because 157 and 142 bp fragments were not clearly separated. Only one mutated allele was found in the group of 221 healthy subjects. This gave an allele frequency of 0.2 (Table I).

Frequency of Duarte Alleles

Duarte galactosemia (Duarte-1 and Duarte-2) is associated with N314D mutation in exon 10 of the GALT gene. N314D mutation was determined by digestion of the amplified fragment (450 bp in length) with *Ava*II restriction endonuclease. A normal allele gives two restriction fragments of 404 and 46 bp. In the case of the homozygote, three fragments were observed for this mutation on stained agarose gel, 302, 102 and 46 bp fragments, as shown in Figure 1b, lane 2. Heterozygote generated four fragments of 404, 302, 102 and 46 bp (Figure 1b, lanes 3, 4). One homozygote and 31 heterozygotes were identified in healthy Croatian population, giving an allele frequency of 7.5 % (D1 + D2 alleles shown in Table I).

To distinguish between D1 and D2 alleles, we searched for the IVS5-24G>A intron variation, which has been reported to be associated with D2 galactosemia and not present in Los Angeles (D1) galactosemia. IVS5-24G>A mutation eliminates the *Sac*I restriction site present in the normal intron 5 of the GALT gene. In the studied group, 19 heterozygotes were found for IVS5-24G>A by identification of specific restriction patterns (fragments of 572, 367 and 215 bp, Figure 1c, lane 1). Two fragments of 367 and 215 bp were observed on stained agarose gel in the absence of mutation (Figure 1c, lanes 3 and 4). In a group of 32 subjects with determined N314D mutation (1 N314D homozygote and 31 N314D heterozygotes), 16 Duarte-2 heterozygotes, 1 Duarte-1 homozygote and 15 Duarte-1 heterozygotes were identified, giving an allele frequency of 3.6 % for D2 and 3.8 for D1 (Table I). Searching other subjects, without N314D mutation, we found another three heterozygotes for the IVS5-24G>A intron variation.

Frequency of K285N Mutation

221 healthy subjects were searched for K285N, which creates additional restriction sites for *Tsp*509I in exon 9 of the GALT gene. The restriction pattern of normal alleles is shown in Figure 1d, lanes 2, 3, 4, including fragments of 573 and 142 bp. No mutated alleles were identified in the studied group of subjects.

Frequencies of Q188R, N314D, and K285N mutations found in healthy population of Croatia were compared with the published data on other healthy populations of

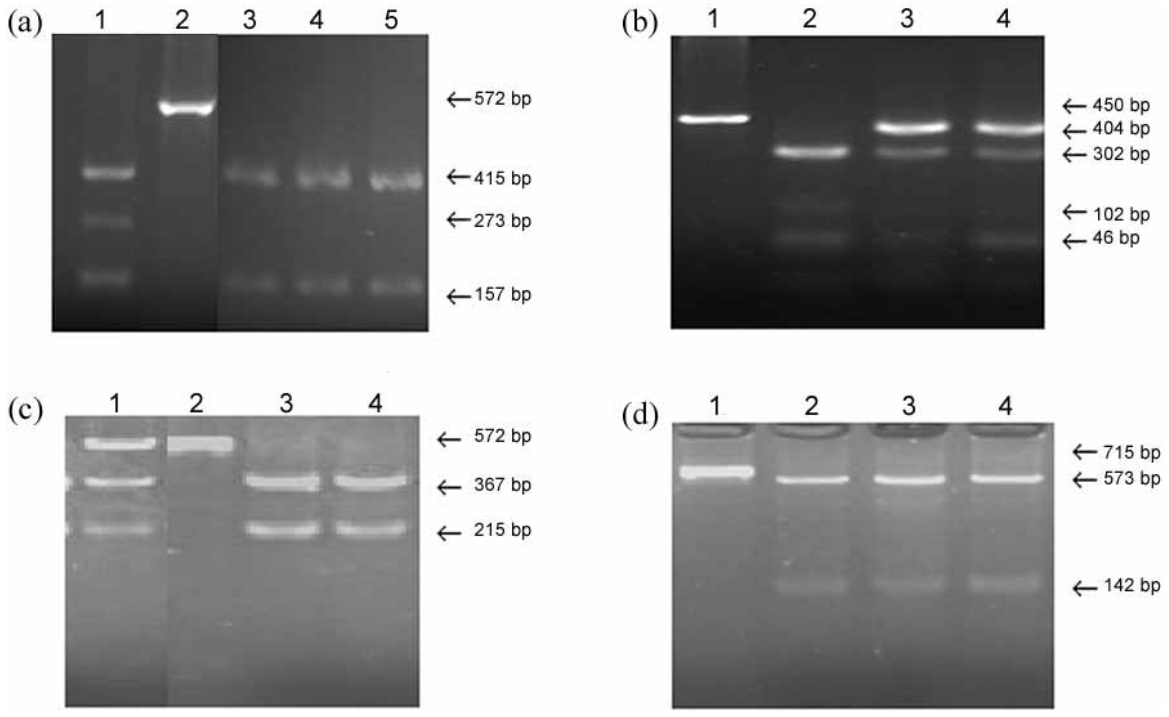

Figure 1. Detection of galactosemia mutations by the RFLP-PCR procedure. (a) Detection of Q188R allele. Lane 1: Q188R heterozygote (QR alleles, fragments of 415, 273 and 157 bp); lane 2: undigested PCR fragment of 572 bp; lanes 3, 4, 5: no Q188R mutation (QQ normal alleles; fragments of 415 and 157 bp). (b) Detection of N314D allele. Lane 1: undigested PCR fragment of 450 bp; lane 2: N314D homozygote (DD alleles, fragments of 302, 102 and 46 bp); lanes 3, 4: N314D heterozygote (ND alleles, fragments of 404, 302, 102 and 46 bp). (c) Detection of IVS5-24G>A allele. Lane 1: VS5-24G>A heterozygote (GA alleles, fragments of 572, 367 and 215 bp); lane 2: undigested PCR fragment of 572 bp; lanes 3, 4: no intron mutations (GG alleles, fragments of 367 and 215 bp). (d) Detection of K285N allele. Lane 1: undigested PCR fragment of 715 bp; lanes 2, 3, 4: no K285N mutation (KK alleles, fragments of 573 and 142 bp).

TABLE II. Allele frequencies of Q188R, N314D, and K285N mutations in different healthy populations. Results of screening healthy population of Croatia for galactosemia mutations are compared with published data on other healthy populations

Region, country, population	*n*	Q188R		N314D (D1 + D2)		N314D-D2		N314D-D1		K285N	
		Allele frequency %	*P*	Allele frequency %	*P*	Allele frequency %	*P*	Allele frequency %	*P*	Allele frequency %	*P*
Croatia	221	0.20		7.5		3.6		3.8		0.0	
Slovenia	174	0.29	0.634 [a]	8.0	0.866 [b]	5.7	0.211 [b]	2.3	0.304 [b]	0.29 [c]	0.683 [a]
Germany	289	-	-	14.9	<0.001 [b]	9.7	<0.001 [b]	5.2	0.388 [b]	-	-
Czech Republic	504	-	-	8.2	0.696 [b]	5.4	0.198	2.8	0.360 [b]	-	-
Italy	802	-	-	7.7	0.933 [b]	3.7	0.932 [b]	4.0	1.000 [b]	-	-
UK	248	-	-	8.9	0.507 [b]	-	-	-	-	-	-
Eastern Europe	254	0.098	0.659 [a]	-	-	-	-	-	-	-	-
Western Europe	1887	0.28	0.859 [a]	-	-	-	-	-	-	-	-
Northern Europe	277	0.90	0.309 [a]	-	-	-	-	-	-	-	-
Southern Europe	176	0.28	0.613 [a]	-	-	-	-	-	-	-	-
USA: average of all populations	4796	0.23	0.704 [a]	7.8	0.870 [b]	5.1	0.201 [b]	2.7	0.197 [b]	0.042	0.433 [a]
USA: Caucasian population	3244	0.29	0.906 [a]	8.8	0.831 [b]	6.0	0.051 [b]	2.8	0.262 [b]	0.062	0.619 [a]

[a] *P* value obtained by Z-test; [b] *P* value obtained by χ-test; [c] Number of cases analyzed for K285N mutation: 346.

Europe and the USA, as shown in Table II.[10,16,18–22] Frequencies of all searched galactosemia mutations in healthy population of Croatia correlate well with those reported for healthy populations of Slovenia, the Czech Republic, Italy, the UK, Eastern, Western, Northern and Southern Europe, and the USA (USA-Caucasian population).[10,16,18–22] There is a statistically significant difference ($p< 0.001$) between Croatian and German populations concerning the N314D mutation, particularly Duarte-2.

Duarte allele is the most frequent galactosemia allele in healthy population of Croatia, followed by Q188R, the classical galactosemia allele. Although heterozygotes for classical and Duarte galactosemia appear to be benign, there are some indications that these mutations increase the risk of developing some diseases, such as ovarian failure and presenile cataracts.[13,14] Results of the presented study provide a basis for further investigation of the association of galactosemia mutations with some diseases. On the other hand, these results seem to be useful for rational neonatal screening for the most frequent galactosemia alleles in Croatia.

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SAŽETAK

Pojavnost mutacija u genu za galaktoza-1-fosfat uridil transferazu u zdravoj populaciji u Hrvatskoj

Karmela Barišić, Lada Rumora, Marija Grdić i Dubravka Juretić

Galaktozemija je bolest uzrokovana nedostatkom jednog od tri enzima koji sudjeluju u metabolizmu galaktoze, galaktokinaze (GALK), galaktoza-1-fosfat uridil transferaze (GALT) ili galaktoza-4 epimeraze (GALE). Nedostatak ili smanjena katalitička aktivnost GALT-a uzroci su klasične galaktozemije. Budući da klasična galaktozemija pokazuje alelnu heterogenost u različitim populacijama i etničkim skupinama, cilj ovog istraživanja bio je utvrditi pojavnost alela za Q188R, N314D i K285N mutacije u zdravoj populaciji u Hrvatskoj. Istraživanje

je obuhvatilo 221 zdravu osobu. Iz periferne krvi izolirana je DNA te utvrđena prisutnost mutacija postupkom koji je obuhvaćao umnožavanje dijela DNA lančanom polimerznom reakcijom nakon koje je slijedila digestija restrikcijskim endonukleazama (PCR-RFLP postupak). Utvrđena je sljedeća pojavnost mutacija: Q188R 0,2 %, N314D 7,5 % te 0 % za K285N. Dobiveni rezultati ne razlikuju se od literaturnih podataka objavljenih za većinu populacija kavkaskoga podrijetla.