Molecular and clinical analysis of patients with classical galactosaemia, galactokinase deficiency and biotinidase deficiency

Ph.D. Thesis

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Budapest, 2010

Introduction

"Nature is no where accustomed more openly to display her secret mysteries than in cases where she shows traces of her workings apart from the beaten path;... For it has been found, in almost all things, that what they contain of useful or applicable is hardly perceived unless we are deprived of them, or they become deranged in some way." William Harvey (1657)

The metabolism is the sum of the chemical processes by which living organized substance is built up and maintained (anabolism), and by which large molecules are broken down into smaller molecules to make energy available to the organism (catabolism). Enzymes are biomolecules that catalyze chemical reactions. An inborn error of metabolism is a genetically determined biochemical disorder in which a specific enzyme defect produces a metabolic block that may have pathological consequences. Serious problems may be prevented if the disorders are discovered early. Newborn screening is the process of testing newborn babies for treatable metabolic diseases.

Three enzyme deficiencies have been described in galactose metabolism. Classical galactosaemia is caused by deficient activity of the enzyme galactose-1-phosphate uridyltransferase (GALT). In the blood of the patients with classical galactosaemia, galactose and galactose-1-phosphate accumulate and are excreted in high concentrations in the urine. Affected infants present in the neonatal period with feeding problems, failure to thrive, vomiting, diarrhea, jaundice, bleeding, liver and renal failure, physical and mental retardation, cataract, neonatal sepsis, which can lead to death. Another form of GALT deficiency is partial galactosaemia associated with about 25% GALT activity. Classical galactosaemia is a monogenic autosomal recessively inherited disorder. The human GALT

gene is located on chromosome 9p13, has a genomic length of 4,3 kb and consists of 11 exons.

Galactokinase deficiency (GALK) is an inborn error of the first step of galactose metabolism. Its major clinical manifestations are the hypergalactosaemia and galactosuria, wich cause the development of cataracts. The disorder is rare worldwide (about 1: 1.000.000), but has a high incidence among the Roma. Galactokinase deficiency is an autosomal recessive disorder. The GALK1 gene is located on chromosome 17q24, spans approximately 7.3 kb of genomic DNA and consists of eight exons.

Biotin is an essential water-soluble vitamin that serves as a coenzyme for four carboxylases in humans. The serum level of biotin depends on the dietary biotin intake and the recycling of endogenous biotin. Biotinidase deficiency (BTD) is an inborn error of biotin recycling that results in biotin depletion. Untreated patients develop various clinical symptoms: neurological deficits, skin rash, alopecia, seizures, ataxia, organic aciduria, hearing loss, metabolic ketoacidosis and occasionally developmental delay. Biotinidase deficiency is classified as either profound or partial based on the serum biotinyl-hydrolase enzyme activity (0–10% and 10–30%, respectively). Biotinidase deficiency is also an autosomal recessively inherited disorder. The BTD gene has been mapped to chromosome 3p25 and consists of four exons.

Early recognition of these three inborn errors and early initiation of the treatment result in rapid clinical improvement. This is made possible by a national newborn screening.

Aims of the study

Our aim was:

- to apply mutation analysis for the whole coding and many regulatory regions of the GALT, GALK1, GALE and BTD genes in the Hungarian setting.

- to identify the genetic cause of classical galactosaemia, galactokinase deficiency and biotinidase deficiency, both retrospectively and prospectively, in all Hungarian patients screened at the Budapest Screening Centre, as well as to identify carriers among family members.

- to describe novel mutations, which provide information about the functional domains of the enzymes and help better understand the structures of the genes.

- to characterize the spectrum of GALT, GALK1 and BTD mutations.

- the clinical characterisation of patients with classical galactosaemia, galactokinase deficiency and biotinidase deficiency and comparison of our results with published data on other populations.

- to analyze genotype-phenotype correlation among all patients screened at the Budapest Screening Centre.

- to analyse the frequencies of the most common galactose-1-phosphate uridyltransferase and biotinidase variant alleles in a healthy Hungarian population.

Methods

- **Patients**

We examined 46 patients with classical galactosaemia, 6 patients with proven galaktokinase deficiency, 60 patients with biotinidase deficiency and 14 patients with high galactose level identified through newborn screening. Consents for mutation analysis were obtained either from the patients or from the parents of patients under 18 years of age.

- **Subjects of the population frequency analysis**

100 healthy subjects (both sexes, over 18 years of age) were screened for frequencies of the most common GALT and BTD mutations. The study was approved by the Ethics Committee. Consents for mutation analysis were obtained from the subjects.

- **Newborn screening for galactosaemia**

Dried blood specimens were tested for galactose. Between 1976 and May 2004, the microbiological method of Guthrie was used. Since 2004, the RA Laboratories Total Galactose Kit was applied for the quantitative measurement of total galactose.

- **Newborn screening for biotinidase deficiency**

Biotinyl-hydrolase activity was determined quantitatively by a colorimetric assay that uses biotinyl-*p*-aminobenzoate (Sigma, Saint Louis, MO) as substrate.

- **DNA isolation from sera and Guthrie card**

DNA was extracted from sera and Guthrie card samples according to the protocol of the High Pure PCR Template Purification kit (Roche Diagnostics GmbH, Mannheim, Germany).

- **PCR reactions**

Each exon and the flanking intron regions of the GALT, GALK1, GALE and BTD gene were PCR-amplified using standard protocols.

- **Direct sequencing**

Direct DNA sequencing was performed on an ABI-PRISM 310 genetic analyser using the BigDye terminator cycle sequencing kit v.3.1 (Applied Biosystems, Foster City, CA).

- **Restriction Fragment Length Polymorphism**

Amplified DNA fragments were digested with Msp I, Tsp509 I, Ava II and Sac I to detect the p.Q188R, p.K285N, p.N314D, and IVS5nt-24G>A polymorphisms, respectively, and with Nla III, Pml I, and BstE II to detect the p.D444H, p.Q456H, and p.A171T mutations, respectively. Restriction products were separated by electrophoresis on a 2% agarose gel.

- **Statistical analysis**

Comparisons of the frequencies of the clinical manifestations were analyzed using the Fischer's exact test. Comparisons of frequencies of the mutations were analyzed using χ^2-test for two independent samples.

Results

I. Classical galactosaemia

I.1. The results of the molecular analysis

Of the 46 patients tested, 13 different sequence variations were identified: 8 missense mutations, 1 nonsense mutation, 1 mutation that destroys the stop codon of the GALT gene and causes elongation of the GALT enzyme's protein chain, and 3 sequence variations with two, five and six variations, respectively, from the normal nucleotide sequence in *cis* (Figure 1).

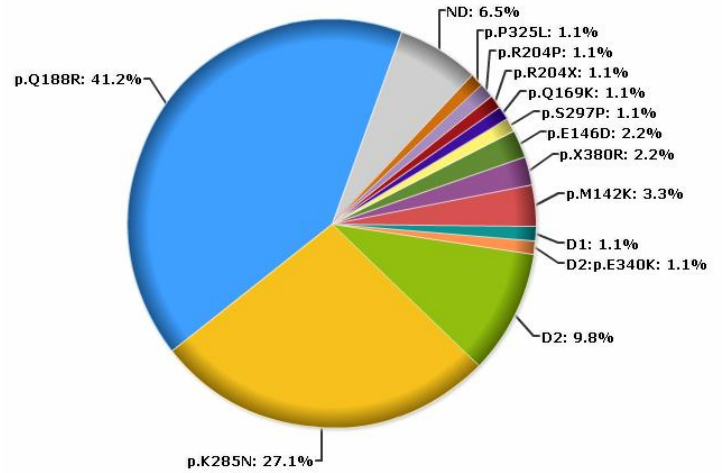

Figure 1. Mutation spectrum of patients with classical galactosaemia (n = 46) and allele frequencies of the mutations

The two most frequent mutations were the p.Q188R and the p.K285N with an allele frequency of 41.2% and 27.1%, respectively. The p.N314D mutation defines two variant forms of the GALT enzyme, the Duarte-1 (D-1) and Duarte-2 (D-2), depending on the presence of additional base changes. The D-2 types are associated with the p.N314D in combination

with 3 intron polymorphisms (IVS4nt-27G>C, IVS5nt+62G>A, IVS5nt-24G>A) and a tetranucleotide deletion in the 5' promoter region (5'UTR-119delGTCA) in *cis*. The D-1 allele was found in 1 patient, while the D-2 allele was found in 9 patients in our population. The p.E340K mutation was detected in addition to all D-2 alterations in *cis* in 1 patient. Two novel (p.S297P and p.E146D) and 6 rare mutations (p.X380R, p.M142K, p.R204X, p.Q169K, p.R204P, and p.P325L) were detected.

I.2. Clinical symptoms

Neonatal symptoms were observed in 46 patients in our Screening Centre. Of them, 31 had no measureable GALT activity, while 15 patients had about 25 percent residual enzyme activity. 29 of the 31 newborns with undetectable GALT activity had severe symptoms in the neonatal period, including feeding difficulties, vomiting, diarrhea, jaundice and hepatomegaly. Most of them required exchange transfusion because of the very high level of galactose-1-phosphate in their blood. Two patients with no GALT activity were the second galactosaemic children in their families, thus, their mothers kept a low-galactose, lactose free diet during pregnancy. Consequently, they had no neonatal symptoms. From the newborns with residual GALT activity, 50% of the patients had mild and 50% of the patients had no neonatal symptoms.

Long-term complications were followed up until 5 years of age in 31 patients. Twenty-five patients had no GALT activity, while 6 patients had residual activity. Of the 25 persons with undetectable enzyme activity 4 patients had cataract (16%), 16 patients (64%) had abnormalities in the motor function, 22 patients (88%) had speech problems (verbal dyspraxia), 5 patients (20%) had severe mental retardation and 7 female patients (58%) had hypergonadotropic hypogonadism. On the other hand, of the 6 patients with about 25 percent GALT activity only one had speech problems.

I.3. The results of the population frequency analysis

The aim of our population study was to analyze a healthy Hungarian cohort for the frequencies of the p.Q188R, p.K285N and p.N314D mutations, and for the Los Angeles (D1) and Duarte (D2) variants. To distinguish between D1 and D2 alleles, we searched for the IVS5nt-24G>A intron variation, which has been reported to be associated with D2 galactosaemia not being present in D1 galactosaemia. No p.Q188R or p.K285N mutant alleles were identified in the studied group of subjects. Allele frequencies for p.N314D mutation, Los Angeles variation and Duarte variation were found to be 11,5%, 2,5% and 9%, respectively.

II. Galactokinase deficiency

II.1. The results of the molecular analysis

We examined the sequence of the human galactokinase gene in 6 patients exhibiting galactokinase deficiency and in 14 additional patients identified at the Budapest Screening Centre as having high galactose level and no reduced GALT activity. We identified two germline mutations.

Six patients were homozygous for the p.P28T missense mutation, the most frequent mutation causing galactokinase deficiency. It was found to account for 83.3% of all abnormal alleles in our population. One patient was homozygous for a novel mutation. The p.L263P missense mutation results in the substitution of a leucin for prolin at position 263 of the GALK protein.

No mutation was detected in the GALK1 and GALE genes in the 14 additional patients with high galactose level and normal GALT activity. However, we identified in this group of the patients a GALK1 promoter (c.-179A>G) and an intronic polymorphism (IVS8nt-40C>T) in intron 7.

II.2. Clinical symptoms

Of the 6 persons found to have galaktokinase deficiency one patient developed cataract prenatally.

III. Biotinidase deficiency

III.1. The results of the molecular analysis

Twenty-four different mutations were identified in 60 patients with biotinidase deficiency. Among these, eighteen were missense mutations (out of these, two were double missense mutations in the same allele), one was a nonsense mutation, two were one-base deletions, one was a seven-base deletion coupled with a three-base insertion, one was an intronic nucleotide change at the splice site of exon 2, and.one was a point mutation that creates a cryptic 3' splice acceptor site (Figure 2).

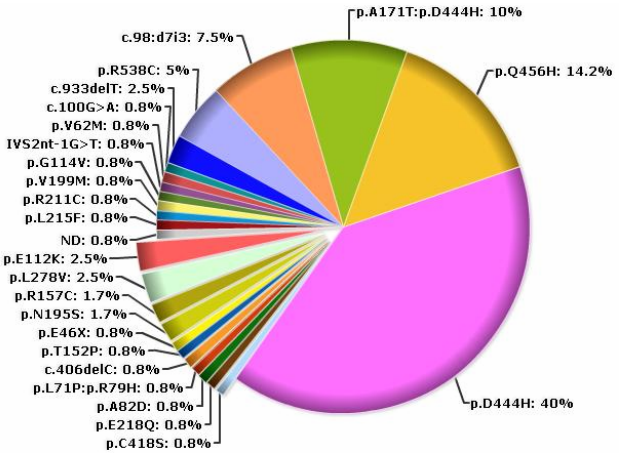

Figure 2. Mutation spectrum of patients with biotinidase deficiency (n = 60) and allele frequencies of the mutations

The 11 mutations protruding from the pie chart were identified in the Hungarian population only

The most frequent mutation in biotinidase deficiency, with 40% allele frequency was the p.D444H mutation, which is the most common cause of partial biotinidase deficiency. The p.Q456H, the p.A171T:D444H, the c.98:d7i3 and the p.R538C mutations were also common among children with biotinidase deficiency identified by newborn screening. These five mutations accounted for 76.7% of all abnormal alleles.

We found 5 different novel mutations in our patients. The p.E46X nonsense mutation results in a termination codon and truncation of the enzyme near the beginning of the translated protein. The p.T152P mutation is a threonine to proline conversion at position 152 of the protein. The p.R157C results in the substitution of an arginine for cysteine at position 157 of the protein. The third novel missense mutation is the p.N195S mutation resulting in a change of asparagine to serine at codon 195. Finally, we found the c.406delC one-base deletion, which is a frameshift mutation.

There were 12 patients with profound biotinidase deficiency in the Western-Hungarian population with a 2.5 ± 4.5% mean biotinyl-hydrolase activity. Of the 12 persons with the profound form of the disease 8 patients had hypotonia (66.67%) and 5 patients (41.67%) had skin rash in the neonatal period. Long-term complications were followed up until 1 year of age (n = 11). All patients had clinical manifestation, especially skin rash (100%). Two patients (18%) had alopecia and 1 patient (9%) had severe allergy.

III.2. Clinical symptoms

All patients with partial biotinidase deficiency (n = 48) were found to be compound heterozygotes for the p.D444H (c.1330G>C) mutation on one allele. The mean biotinyl-hydrolase activity in this patient group was 19.6 ± 9.3%. Of the patients ascertained by routine newborn screening (n = 47) 53.19% showed skin problems and 6.38% had alopecia. Long-term complications were followed up until 1 year of age (n = 47). Of all patients

with partial biotinidase deficiency 53.19% had skin rash and eczema, 6.38% had alopecia and 10.6% had allergy.

III.3. The results of the population frequency analysis

One hundred healthy subjects were screened for the p.D444H, p.Q456H and p.A171T:p.D444H mutations to assess the Hungarian population frequencies of these most common BTD mutations. The p.A171T mutation was only screened for in p.D444H carriers, because it always presents as a double mutation. No p.A171T mutant alleles were identified in the studied group of subjects. Allele frequencies for p.D444H and p.Q456H mutations were found to be 5.5% and 0.5%, respectively.

Conclusions

- The incidence of classical galactosaemia is 1 in 47.480 in Western Hungary, a frequency similar to that in most European countries.
- 13 different mutations were identified in 46 patients with classical galactosaemia including two novel missense mutations (p.S297P; p.E146D).
- The p.Q188R was found to be the most common mutation in the Western Hungarian population (allele frequency: 41.2%). This result fits in the observations that the relative p.Q188R mutation frequency decreases from West to East and from North to South in the European continent. Although the p.K285N mutation is a relatively infrequent mutation globally, it is the second most common disease-causing mutation in some countries in Eastern-Central Europe, including Hungary. We detected this mutation with a 27.1% allele frequency. This alteration, unlike p.Q188R, shows an increasing gradient in frequency across the continent towards the East.
- We described a potentially complementary effect on GALT enzyme activity of the p.N314D and p.E304K codon changes when present in *cis*.
- The galactokinase deficiency is rare worldwide, but has a high incidence among the Roma. The incidence of the disorder in Western Hungary supports this observation.
- The p.P28T was found to be the most common GALK1 mutation in the Western Hungarian population, which is a founder Romani mutation. We identified the p.L263P novel mutation.
- The incidence of the biotinidase deficiency in Western Hungary (1:18,850) is more than three times the worldwide incidence.

- Twenty-four different mutations were identified in 60 patients with biotinidase deficiency.
- Similar to the worldwide incidence, the five most common BTD mutations were: p.D444H, p.Q456H, c.98:d7i3, p.A171T:p.D444H and p.R538C.
- We identified five novel mutations (p.E46X; p.T152P; p.R157C; p.N195S; c.406delC) in the biotinidase gene.
- A relatively high proportion of mutations (14.2%) proved to be unique to the Hungarian population.
- In monogenic autosomal recessive disorders the clinical manifestations do not always correlate closely with the genotypes. Patients with identical genotypes showed extremely variable phenotypes. The symptoms depend on other genetic and environmental factors.
- The allele frequencies of the most frequent galactose-1-phosphate uridyltransferase alleles in the Hungarian population correlate well with other healthy Caucasian populations.
- The allele frequencies of the most biotinidase variant alleles in the Hungarian population are usually higher than in other healthy Caucasian populations.

Publications

Related publications:

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5. Somogyi Cs, Kiss E, **Milánkovics I.** (2004) Mi a galaktozémia? Phare kiadvány

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