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A Common Mutation Associated with the Duarte Galactosemia Allele

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Summary

The human cDNA and gene for galactose-1-phosphate uridyl transferase (GALT) have been cloned and sequenced. A prevalent mutation (Q188R) is known to cause classic galactosemia (G/G). G/G galactosemia has an incidence of 1/38,886 in 1,396,766 Georgia live-born infants, but a more common variant of galactosemia, Duarte, has an unknown incidence. The proposed Duarte biochemical phenotypes of GALT are as follows: D/N, D/D, and D/G, which have ~75%, 50%, and 25% of normal GALT activity, respectively. In addition, the D allele has isoforms of its enzyme that have more acidic pI than normal. Here we systematically determine (*a*) the prevalence of an A-to-G transition at base pair 2744 of exon 10 in the GALT gene, a transition that produces a codon change converting asparagine to aspartic acid at position 314 (N314D), and (*b*) the association of this mutation with the Duarte biochemical phenotype. The 2744G nucleotide change adds an *Ava*II (*Sin*I) cut site, which was identified in PCR-amplified DNA. In 111 biochemically unphenotyped controls with no history of galactosemia, 13 N314D alleles were identified (prevalence 5.9%). In a prospective study, 40 D alleles were biochemically phenotyped, and 40 N314D alleles were found. By contrast, in 36 individuals known not to have the Duarte biochemical phenotype, no N314D alleles were found. We conclude that the N314D mutation is a common allele that probably causes the Duarte GALT biochemical phenotype and occurs in a predominantly Caucasian, nongalactosemic population, with a prevalence of 5.9%.

Introduction

Galactose-1-phosphate uridyl transferase (GALT) catalyzes the second step of the Leloir pathway of galactose metabolism in which galactose-1-phosphate and UDP-glucose form UDP-galactose and glucose-1-phosphate (Leloir 1957). GALT functions as a dimer and is highly conserved in evolution, and the two-step process it catalyzes is thought to exhibit ping-pong kinetics (Frey et al. 1982). Classic (transferase) galactosemia is a disease of galactose metabolism in which the activity of GALT is deficient (Segal 1989). Untreated infants with classic galactosemia often present with failure to thrive, jaundice, hepatosplenomegaly, cataracts, *Escherichia coli* sepsis, and perhaps even death, within a few days of exposure to milk. Population-based newborn screening with elimination of lactose from the diet will prevent these symptoms. However, the long-term outcome may include cataracts, speech defects, premature ovarian failure, growth decline, and poor intellectual function (Donnell et al. 1961; Komrower and Lee 1970; Kaufman et al. 1981; Waisbren et al. 1983; Waggoner et al. 1990).

In classic galactosemia, there is virtually no GALT activity detected. In addition to the classic form, however, there are other variant forms of the enzyme that have diminished but detectable activity (Segal 1989). One such variant is the Duarte (D) allele, first described by Beutler (Beutler et al. 1965, 1966). The D allele was defined biochemically by reduced enzyme activity and by an isoform distinguishable by gel electrophoresis and isoelectric focusing (Kuhnl et al. 1974; Kelley et al. 1983; Shin et al. 1987; Sparkes et al. 1987; Kelley and Segal 1989). Heterozygotes for the normal and Duarte alleles (N/D) are presumed to have 75% normal GALT

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activity, homozygotes (D/D) to have 50% activity, and compound heterozygotes for the Duarte allele and the classic galactosemia allele (D/G) to have 25% activity, in peripheral erythrocytes. At least seven families have been reported with GALT isoforms and immunochemical reactions identical to those of Duarte, except for increased activity when compared with controls. Those families were defined only in California and Mexico, and the allele is called the "Los Angeles variant" (Ng et al. 1973; Ibarra et al. 1979; Anderson et al. 1984). N/D and D/D babies are usually asymptomatic in the neonatal period. D/G newborns may exhibit some symptoms and may have accumulation of precursor substrates of the blocked reaction (Greenberg et al. 1989). The long-term effects of carrying the Duarte allele, on older children and adults, are unknown.

The cloning and sequencing of the GALT cDNA and gene made possible more sensitive and specific screening techniques for variation in GALT (Reichardt and Berg 1988; Flach et al. 1990; Leslie et al. 1992). The gene is ~4 kb long and is organized into 11 exons (Leslie et al. 1992). There is high sequence homology in evolution through three species (yeast, *E. coli,* and human), with small regions of absolute identity (Reichardt and Berg 1988; Flach et al. 1990). A mutation in exon 6 of the GALT gene was found that converted the codon for glutamine at position 188 to an arginine (Q188R) (Reichardt et al. 1991; Leslie et al. 1992; Elsas et al. 1993; Fridovich-Keil and Jinks-Robertson 1993). The Q188R mutation has a prevalence of ~70% in a Caucasian G/G population (Elsas et al. 1993). Patients who are homozygous for this mutation have essentially no detectable GALT activity (Fridovich-Keil and Jinks-Robertson 1993). When the Q188R mutation is expressed in transformed yeast, there is no GALT activity (Fridovich-Keil and Jinks-Robertson 1993).

In initial studies of abnormal GALT genes, a base-pair transition was found converting an A to a G at position 2744 of exon 10. This produced a missense codon converting an asparagine to aspartic acid at amino acid position 314 (N314D) (Reichardt and Woo 1991; Leslie et al. 1992). In preliminary studies without confirmatory biochemical phenotypes, we found the N314D change in six of six patients said to have the Duarte or Los Angeles biochemical phenotypes and in 6 of 40 nonphenotyped control alleles (Leslie et al. 1992). These findings suggested that the N314D sequence change was associated with the Duarte allele.

In the present study, we systematically determine the prevalence of the N314D genotype in individuals whose biochemical phenotype with respect to the Duarte GALT variant is known, compare this prevalence with that found in biochemically nonphenotyped controls, and analyze the effect of these mutant alleles on erythrocyte GALT in genotyped patients.

Material and Methods

Enzyme and Biochemical Reagents

The restriction endonucleases *Ava*II and its isoschizomer, *Sin*I, were purchased from Promega. ^{14}C-galactose-1-phosphate was purchased from DuPont/New England Nuclear. Oligonucleotide primers were synthesized on an Applied Biosystems oligonucleotide synthesizer at the Emory Microchemical Facility.

Patient Samples

Genomic DNA from white blood cells and lymphoblastoid cell lines were obtained from galactosemia patients cared for through the Division of Medical Genetics, Department of Pediatrics, Emory University School of Medicine. All patients had biochemical GALT phenotypes determined enzymologically in our laboratory. Genomic DNA was also taken from white cells of 111 nongalactosemic individuals, including healthy donors, diabetics, cystic fibrosis patients, and normal women having amniocentesis. These patients' GALT was not biochemically phenotyped. Of the 90 controls for whom ethnic backgrounds were known, 24 were of African American descent, and 66 were Caucasian. Patients and families gave informed consent to participate in this project, and the research project was approved by the Institutional Review Board at Emory University.

Red Cell GALT Assay

Red cell GALT activity was measured by assaying the conversion of ^{14}C-galactose-1-phosphate and UDP-glucose to glucose-1-phosphate and ^{14}C-UDP-galactose at 37°C. Reaction products were separated by descending DEAE paper chromatography and were counted in a scintillation counter by using modifications of methods described elsewhere (Henry 1964; Mellman and Tedesco 1965; Ng et al. 1967; Lee and Ng 1982).

GALT Isozyme Assay

GALT was purified from red cell lysates on a DEAE-Sephacel column, was concentrated, and then was electrophoresed on an isoelectric-focusing gel and was stained for enzyme activity. The Duarte allele demonstrated migration of bands toward the anode and the pH 5.0 gradient by isoelectric gel focusing. These procedures utilized methods that have been published else-

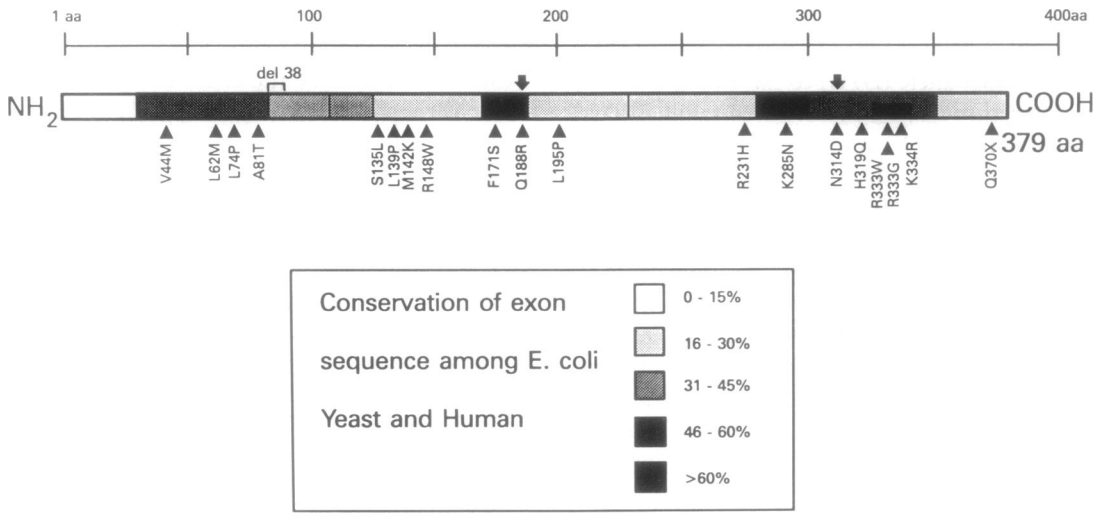

Figure 1 Evolutionary conservation and codon changes of the GALT protein sequence. Conservation of exon sequences from *Escherichia coli*, yeast, and humans is indicated by increasingly dark shading with the range of conserved sequence indicated as percent. Exonic coding sequences are delineated, and the two prevalent mutations are indicated by arrows.

where (Kuhnl et al. 1974; Kelley et al. 1983; Shin et al. 1987; Sparkes et al. 1987).

DNA Analysis

The N314D sequence change was identified by PCR and restriction endonuclease cutting. This A-to-G transition introduces a new *Ava*II endonuclease restriction site: TGGAACCAT→TGGGACCAT. We amplified the DNA for 30 cycles of denaturation at 94°C for 1 min, reannealing at 58°C for 30 s, and extension at 72°C for 1 min. Primer sets for intron G (5′) CGCGAATTCCCTTGCCTATTTGCTGACCAC and intron J (3′) GGGGTCGACGCCTGCACATACTGCATGTGA amplify a 949-bp segment of DNA including exons 8–10 of the human GALT gene.

The Q188R sequence change was identified using the following primer set for amplification by PCR: primer 1 (5′)- GGGTCGACGTCGGATGTAACGCTGCCACTCA-(3′) and primer 2 (5′)- GGGTCGACTAGCTCCTGGCGGCTGTACTCCA-(3′). Conditions for amplification by cyclical denaturation, primer annealing, and DNA extension have been reported elsewhere (Elsas et al. 1993). The A- to-G transition in exon 6 at codon 188 introduces a new *Hpa*II cut site, which was used to determine molecular genotype in this population.

Results

By inspection the N314D codon change is a good candidate to produce the partial enzymatic impairment associated with the Duarte biochemical phenotype. The N314D codon change is situated near the carboxy terminus of the deduced GALT protein sequence and is encoded in exon 10 of the human GALT gene (fig. 1). The asparagine 314 codon is not as highly conserved as the glutamine 188 of exon 6 (fig. 1; Flach et al. 1990; Leslie et al. 1992).

The Duarte biochemical phenotype was assigned to patients if bands 5 and 6 were prominent using a GALT isozyme analysis by isoelectric focusing (fig. 2). Although total GALT activity in erythrocytes was also analyzed, it was not consistently reduced in the D/N biochemical phenotype, but it was reduced in the D/D and D/G biochemical phenotypes. Assignment of biochemical phenotype was occasionally assisted by analysis of first-degree relatives.

The presence or absence of the N314D codon change was then determined without prior knowledge of the biochemical phenotype, on the basis of the presence or absence of a new *Ava*II cut site in the 949-bp amplified fragment representing genomic sequence from exons 8–10 (fig. 3). In this amplified nucleotide sequence, there were four constitutive *Ava*II restriction sites producing five fragments, of 581, 164, 92, 82, and 30 bp. A fifth *Ava*II site was created in the 581-bp fragment if the A-to-G transition at bp 2744 (N314D) were present, thereby producing 479- and 102-bp fragments in place of the 581-bp piece. Genotyping for the N314D codon change was accomplished as outlined in

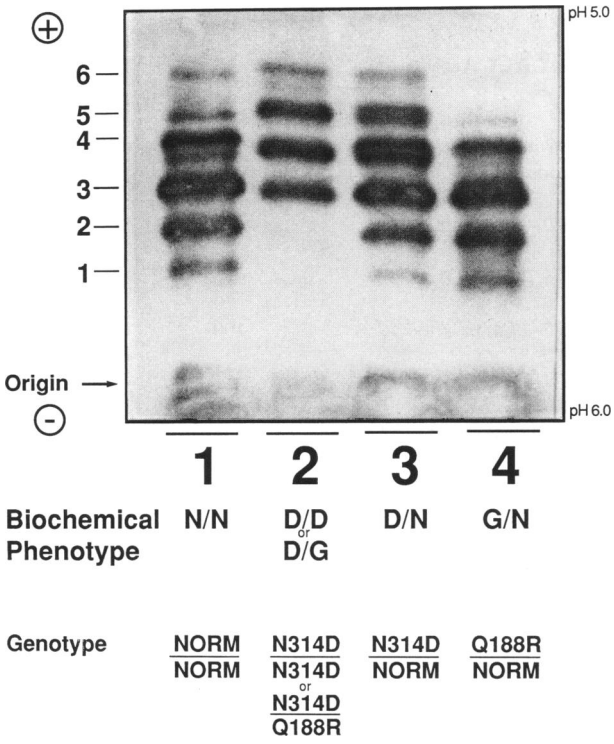

Figure 2 Agarose isoelectric-focusing gel of erythrocyte GALT, showing the migration patterns of the following biochemical phenotypes: N/N, D/D or D/G, D/N, D/D, and G/N. The Duarte allele demonstrates more intense bands, which migrate to the anode and pH 5. These bands are designated "5" and "6." The biochemical phenotypes and postulated molecular genotypes are designated beneath each lane. D/G and D/D are differentiated by finding ~75% and ~50% reduction in GALT activity, respectively, and by pedigree analysis.

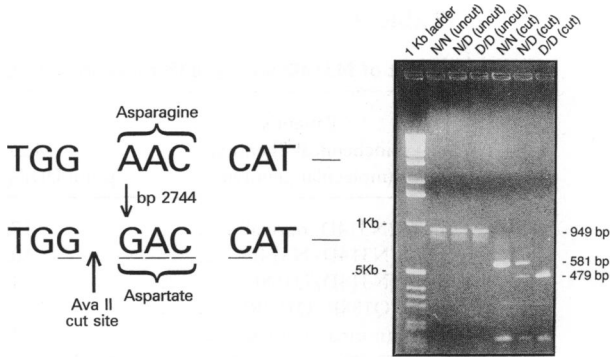

Figure 3 Molecular strategy for screening of the N314D sequence change. *Left,* Description of the A-to- G transition with appearance of a new *Ava*II cut site. *Right,* Genotyping using amplification and *Ava*II restriction-endonuclease cutting. Each lane of this 1% agarose gel is labeled with the biochemical phenotype of the donor's DNA above. The fragment sizes are indicated to the right.

the right-hand panel of figure 3. Absence of the N314D change produced only the 581-bp sequence, while heterozygotes for N314D had both the 581- and 479-bp fragments. The homozygous N314D genotype had the 479-bp fragment and lacked the 581-bp fragment.

We then used this method to screen three populations. The first included 111 patients (222 alleles) whose DNAs were available but who had neither galactosemia nor previous determination of biochemical phenotypes for their GALT enzyme(s) (table 1). Approximately 73% were Caucasian and ~27% were of African American descent. Of these 222 alleles, 13 had the N314D codon change, for a prevalence of 5.9%. We then prospectively analyzed DNA of patients whose erythrocyte DNA was biochemically characterized either as having the Duarte variant or not. Of the 37 patients, 34 were heterozygous and 3 were homozygous for the D enzyme isoform (40 D alleles). Of these 40 D alleles identified biochemically, 40 N314D alleles were identified when analyzed separately and blindly. Conversely, among 36 control patients (72 normal alleles) whose erythrocytes did *not* have reduced GALT activity or the Duarte isoform, no N314D codon changes were found (table 1).

We analyzed GALT activity on fresh blood specimens from fully genotyped patients and controls to determine the effect of the codon sequence changes on the GALT activity in their peripheral erythrocytes (table 2). Three homozygotes for N314D (D/D) and 13 compound heterozygotes for Q188R/N314D (D/G) had GALT activities lower than control values and consistent with postulated effects on GALT activity. How-

Table 1

Prevalence of the N314D Allele in Biochemically Phenotyped and Nonphenotyped Populations

Population	No. of Alleles	No. of N314D	Prevalence (%)
Nonphenotyped[a]	222	13	5.9
Phenotyped:[b]			
Duarte	40	40	100.0
Not Duarte	72	0	.0

[a] One hundred eleven predominantely Caucasian patients with no history of galactosemia.

[b] Thirty-four patients with D/N or D/G and three with D/D GALT phenotypes. Thirty-six patients had no biochemical evidence of the D allele.

Table 2

Effect of N314D and Q188R Mutations on Erythrocyte GALT Activity

Patient's Biochemical Phenotype (molecular genotype)	Mean GALT Activity ± SD (range) (μmol UDP-galactose produced/h/g hemoglobin)	% of normal/normal
D/N (N314D/normal)	17.5 ± 4.8 (13.0–27.8) (n = 8)	74
D/D (N314D/N314D)	10.3 ± .7 (n = 3)	43
D/G (N314D/Q188R)	3.7 ± 1.4 (.4–5.7) (n = 13)	16
G/G (Q188R/Q188R)	.06 ± .03 (.00–.40) (n = 7)	0
N/N (normal/normal)	23.8 ± 4.7 (16.7–29.4) (n = 8)	100

ever, individuals with the N314D/normal (D/N) genotype had GALT activity that was not statistically different from those of our eight genotyped controls. No GALT activities greater than normal (presumed Los Angeles variants) were identified in this population, which did not include patients of Hispanic descent (table 2).

Discussion

If the N314D sequence change does cause the Duarte biochemical phenotype, it should occur with increased frequency in the affected population compared with a control population. In addition, it should occur in a region of the gene likely to produce only partial enzyme impairment. The results from figure 1 and tables 1 and 2 conform to these requirements. We found the prevalence of the N314D sequence change in a nongalactosemic control group with unknown GALT phenotype to be 5.9%. By contrast, the prevalence was 100% among patients found to have a Duarte allele after we decoded a blinded biochemical analysis of their GALT enzymes. In a prospective study of subjects who were known not to have the Duarte biochemical phenotype, the N314D sequence change was not detected. We conclude from these prevalence values that the N314D sequence change does not occur at random, but is found with an extraordinary concordance in a subset of galactosemia patients exhibiting the Duarte biochemical phenotype. Furthermore, the prominent isoform bands in the Duarte GALT protein move toward the anode by electrophoresis and toward the lower pH by isoelectric focusing, a finding consistent with the substitution, of a negatively charged aspartate for asparagine, found in the N314D allele.

What is the frequency of the N314D allele? Published estimates of the frequency of the Duarte biochemical phenotype in the general population range from 4% to 14% (Mellman et al. 1968; Tedesco 1972; Beutler 1973; Gitzelmann 1980; Fox 1987; Greenberg et al. 1989). We found a frequency of the N314D allele of 5.9% in a heterogeneous population of nongalactosemic patients consisting of ~73% Caucasians and ~27% African Americans, a frequency that is within the range previously predicted by biochemical screening. Population screening for the N314D allele can now be performed to obtain a more accurate estimate and to assess its possible association with such chronic disorders of galactosemia as cataracts, premature ovarian failure, speech defects, and growth restriction.

We evaluated the possibility that N314D may itself be a polymorphism and that the reduced GALT activity often observed in Duarte patients reflected other causes. The 11 GALT gene exons in the three homozygotes for N314D contained no other codon changes when screened for SSCPs (table 2). Others have suggested that the N314D mutation is also related to the Los Angeles allele, a variant that displays the D allele isoforms but that is associated with increased rather than decreased GALT activity (Reichardt et al. 1993). When these investigators studied the N314D sequence in a mammalian expression system, a moderate (though not significant) increase in GALT activity was found (Reichardt and Woo 1991). The COS monkey cells have abundant endogenous GALT enzyme, so heterodimers (N314D/normal monkey GALT) might occur as well as homodimers (N314D/N314D and monkey GALT/monkey GALT). It is difficult to reconcile these findings with our analysis of human erythrocytes. Three N314D/N314D homozygotes had 43% of the median value for N/N homozygotes (P<.001) (table 2). One possible interpretation of these data is that the Duarte biochemical phenotype as observed in humans reflects some subtle effect that is not faithfully reproduced in the model expression system involving COS cells. Candidate mechanisms for our observed partial

impairment of GALT by N314D could involve species-specific codon bias impacting GALT protein translation levels, assembly, function, and/or degradation of the mature dimeric proteins. Our results clearly demonstrate a striking association of the N314D mutation with the Duarte biochemical phenotype and strongly suggest that this association is causal. It is also possible that the Los Angeles variant either does not exist or results from either another allelic variation in the GALT gene or perhaps epigenetic phenomena found in the Hispanic population. Questions concerning the structural biology of the GALT enzyme can be approached both through further studies of transformed expression systems containing identified variations in the GALT nucleotide sequence and through studies of GALT gene expression, posttranscriptional processing, and protein synthesis and degradation in diploid human cells.

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