A Case Study of Monozygotic Twins Apparently Homozygous for a Novel Variant of UDP-Galactose 4'-epimerase (GALE)

A Complex Case of Variant GALE

Ying Liu • Kristi Bentler • Bradford Coffee •
Juliet S. Chhay • Kyriakie Sarafoglou •
Judith L. Fridovich-Keil

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Abstract Epimerase deficiency galactosemia is an autosomal recessive disorder that results from partial impairment of UDP-galactose 4'-epimerase (GALE), the third enzyme in the Leloir pathway of galactose metabolism. Clinical severity of epimerase deficiency ranges from potentially lethal to apparently benign, likely reflecting the extent of GALE enzyme impairment, among other factors. We report here a case study of monozygotic twins identified by newborn screening with elevated total galactose and normal galactose-1P uridylyltransferase (GALT). Follow-up testing revealed partial impairment of GALE in hemolysates but near-normal activity in lymphoblasts; molecular testing identified a missense substitution, R220W, apparently in the homozygous state. The twins were treated with dietary galactose restriction for the first 18 months of life. During this time, independent testing revealed concurrent diagnoses of Williams Syndrome in both twins, and cytomegalo-

virus (CMV) infection in one. Expression studies of R220W-hGALE in a null-background strain of *Saccharomyces cerevisiae* demonstrated a very limited impairment of $V_{\max}$ for UDP-galactose (UDP-Gal) and K_m for UDP-N-acetylgalactosamine (UDP-GalNAc), but a galactose challenge in vivo failed to uncover any evidence of impaired Leloir function. Similarly, both twins demonstrated normal hemolysate galactose-1-phosphate (Gal-1P) levels following normalization of their diets at 18 months of age. While these studies cannot rule out a negative consequence from some cryptic GALE impairment in a specific tissue or developmental stage, they suggest that the substitution, R220W, is mild to neutral, so that any GALE impairment in these twins is likely to be peripheral and therefore unlikely to be the cause of the negative outcomes observed.

Abbreviations

GALE	UDP-galactose 4'-epimerase
GALT	Galactose-1-phosphate uridylyltransferase
UDP-Gal	Uridine diphosphate galactose
UDP-GalNAc	Uridine diphosphate-N-acetyl-D-galactosamine
UDP-Glc	Uridine diphosphate glucose
UDP-GlcNAc	Uridine diphosphate-N-acetyl-D-glucosamine

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Y. Liu • J.S. Chhay • J.L. Fridovich-Keil (✉)
Department of Human Genetics, Emory University, School of
Medicine, Room 325.2 Whitehead Building, 615 Michael Street,
Atlanta, GA 30322, USA
e-mail: jfridov@emory.edu

K. Bentler • K. Sarafoglou
Division of Genetics and Metabolism, Department of Pediatrics,
University of Minnesota Amplatz Children's Hospital,
Minneapolis, MN 55455, USA

B. Coffee
Division of Medical Genetics, Department of Human Genetics,
Emory University, School of Medicine, Atlanta, GA 30322, USA

Introduction

The galactosemias are a family of genetic disorders that result from impaired ability to metabolize galactose; these autosomal recessive conditions are caused by mutations that compromise either the expression or function of

galactokinase (GALK, EC 2.7.1.6), galactose-1-phosphate uridylyltransferase (GALT, EC 2.7.7.12), or UDP-galactose 4'-epimerase (GALE, EC 5.1.3.2), the three enzymes of the Leloir pathway (Fridovich-Keil and Walter 2008). The symptoms and severity of these conditions vary in response to which enzyme is impaired and the extent of the impairment; other genetic and environmental factors remain poorly understood.

Epimerase deficiency (MIM# 230350), also called Type III galactosemia, results from partial impairment of GALE and is perhaps the least well understood of the galactosemias. Fewer than 10 patients with severe GALE impairment, resulting in a clinical presentation similar to that of classic transferase deficiency galactosemia, have been reported (Holton et al. 1981; Sarkar et al. 2010; Walter et al. 1999); these patients are said to have generalized epimerase deficiency (Fridovich-Keil et al. 2011). Similar to patients with classic galactosemia, patients with generalized epimerase deficiency respond well to dietary restriction of galactose, which prevents the potentially lethal acute symptoms, although long-term complications may persist (Henderson and Holton 1983; Sarkar et al. 2010; Walter et al. 1999).

Most patients with epimerase deficiency are asymptomatic in infancy and are identified through newborn screening in jurisdictions that measure both GALT activity and total galactose in every sample; the blood spots from these infants show elevated total galactose despite normal GALT (Fridovich-Keil et al. 2011). Follow-up enzymatic testing of hemolysates from patients with epimerase deficiency galactosemia demonstrates partial to profound loss of GALE activity. Enzymatic testing of fibroblasts or transformed lymphoblasts reveals a broad range of impairment from essentially normal GALE activity down to 15–20% of normal (Gitzelmann and Steimann 1973; Gitzelmann et al. 1976; Mitchell et al. 1975; Openo et al. 2006). Individuals with normal or near-normal GALE activity in cell types other than red blood cells are said to have peripheral epimerase deficiency, and are believed to remain asymptomatic (Fridovich-Keil et al. 2011; Gitzelmann et al. 1976). Individuals who show partial impairment of GALE activity in cell types other than red blood cells are said to have intermediate epimerase deficiency (Openo et al. 2006); the long-term outcomes for these patients remain unknown as nearly all are lost to follow-up at an early age (Alano et al. 1998; Fridovich-Keil et al. 2011; Quimby et al. 1997; Wohlers et al. 1999).

More than 20 different, ostensibly causal genetic variants have been identified in the GALE loci of patients with biochemically confirmed epimerase deficiency; a subset of these alleles and the GALE proteins they encode have been studied in vitro and/or in vivo, revealing a range of degrees and mechanisms of impairment (Alano et al. 1998; Bang

et al. 2009; Chhay et al. 2008b; Henderson et al. 2001; Maceratesi et al. 1996, 1998; Openo et al. 2006; Park et al. 2005; Quimby et al. 1997; Thoden et al. 2001b; Timson 2005; Wohlers and Fridovich-Keil 2000; Wohlers et al. 1999). Some variant GALE proteins are catalytically impaired, while others demonstrate compromised stability, at least under defined laboratory conditions. While the relationship between GALE activity and galactose-sensitivity has been defined in yeast and *Drosophila* models (Sanders et al. 2010; Wasilenko and Fridovich-Keil 2006), the relationship between GALE activity level and outcome severity in patients remains a point of speculation.

We report a case study on the discordant clinical, biochemical, and genetic phenotypes of monozygotic twins with partial GALE deficiency identified by newborn screening on the basis of normal GALT and elevated total galactose levels. Both twins were apparently homozygous for a novel variant of GALE (R220W) and had multiple concurrent abnormalities including severe vitamin D-deficiency rickets, moderate bilateral sensorineural hearing loss, and genomic deletions consistent with Williams syndrome. Our report illustrates the complexity of comorbidities that can exist in a single patient and serves as a reminder that not every genetic variant is causal of the clinical abnormalities present.

Materials and Methods

Study subjects: The twins, designated here as FKE065 and FKE066, were ascertained by referral from their metabolic nurse practitioner; informed consent was obtained in accordance with Emory University Institutional Review Board Protocol 618–99. Control samples were ascertained as anonymous blood samples from nongalactosemic individuals, also in accordance with IRB protocol 618–99. The hemolysate biochemical data listed here were generated in clinical labs, as noted.

Mutational analysis of the *hGALE* locus: Mutational analysis of the *hGALE* loci in both twins was performed in a CLIA-approved clinical laboratory by direct sequencing of PCR-amplified fragments of genomic DNA representing all coding exons and their immediate flanking intronic sequences. Each amplicon was sequenced in both the forward and reverse directions and nucleotide changes were interpreted using the Human Genome Mutation Database (HGMD) and dbSNP database. The functional significance of the R220W substitution in *hGALE* was estimated using three software systems: SIFT (<http://sift.jcvi.org/>, Ng and Henikoff 2003), PANTHER (Thomas et al. 2003), and Polyphen 2 (<http://genetics.bwh.harvard.edu/pph2/>, Adzhubei et al. 2010).

Lymphoblast studies: EBV-transformed lymphoblasts were prepared from patient and control blood samples as described previously (Neitzel 1986). Transformed lymphoblasts were

maintained in RPMI-1640 medium (Hyclone) containing glucose (2 g/L) and L-glutamine (0.3 g/L) and supplemented with penicillin (100 U/mL), streptomycin (100 mg/mL), 25 mmol/L Hepes, and 10% (v/v) fetal bovine serum (FBS) (Gibco/Invitrogen, Carlsbad, CA, USA). All cells were maintained at 37 °C in a humidified 5% CO₂ incubator (NuAire, Plymouth, MN, USA).

GALT and GALE enzyme analyses of lymphoblast cell lysates: Lymphoblast cell cultures were harvested and lysates prepared and analyzed for GALT and GALE activities as described previously (Openo et al. 2006). Enzyme activity was defined in units of pmol product produced/min/μg soluble protein.

Plasmids and yeast strains: The R220W substitution was re-created by site-directed mutagenesis of a wild-type human GALE coding sequence within the context of a centromeric yeast expression plasmid (MM33) that has been previously described (Chhay et al. 2008b). Mutagenesis was carried out using the Quick-change system (Stratagene, Inc.) according to the manufacturer's instructions using the following primer sequences (lower case letters indicate the mutation to be created): hGALE.R220W.fl 5' GTGGCGATCGGGC-GATGGGAGGCCCTGAATGTC 3' and hGALE.R220W.r1 5' GACATTCAGGGCCTCCCaTCGCCCCGATCGCCAC 3'. The entire hGALE open reading frame of the resulting plasmid (pMM33.hGALE.R220W) was confirmed by dideoxy sequencing. The corresponding positive (wild-type hGALE) and negative (plasmid backbone only) plasmid controls have been reported previously (Chhay et al. 2008b; Wohlers et al. 1999).

All yeast strains used in this study were derived by transformation of JFy3835, a *GAL10*-null haploid strain of *Saccharomyces cerevisiae* that lacks endogenous GALE and has been described previously (Chhay et al. 2008b). For in vitro biochemical assays, yeast cultures were maintained in standard synthetic medium containing 2% dextrose (SD) at 28 °C. For growth curves, yeast were cultured in standard synthetic medium containing 2% glycerol and 2% ethanol (SGE) at 30 °C, with or without the addition of galactose, as indicated.

Enzyme assays of yeast soluble lysates: Yeast proteins were extracted from cells harvested from SD cultures at OD₆₀₀ between 0.8 and 1.2 by vigorous agitation with glass beads, as previously described (Chhay et al. 2008b). Soluble lysates were passed over P-6 Bio-Spin columns (Bio-Rad) to remove small metabolites prior to further analysis. The protein concentration in each sample was determined using the Bio-Rad protein assay reagent as recommended by the manufacturer using a standard curve of BSA. GALE and GALT enzyme activities were measured as described previously (Chhay et al. 2008b). Kinetic studies of each protein were performed in triplicate over a range of five different concentrations of substrate

(UDP-Gal or UDP-GalNAc) with NAD⁺ held constant, at 0.5 mM, and also in triplicate over a range of five different concentrations of NAD⁺ with substrate held constant (UDP-Gal at 0.8 mM or UDP-GalNAc at 0.66 mM). Data were analyzed by SigmaPlot 11.0 software using the ligand-binding plot of the Michaelis–Menten equation ($1/V = K_m/(V_{\max} [S]) + 1/V_{\max}$).

Yeast growth assays in the presence of galactose: Yeast growth assays were performed as described previously (Chhay et al. 2008a).

Statistical analyses: Statistical analyses were performed using the JMP 8.0.1 software package. Data were analyzed using linear regression, one-way ANOVA, or *t*-test. The results were considered statistically significant if *P* < 0.05.

Case History

Infants FKE065 and FKE066 were delivered from a diamniotic dichorionic twin pregnancy in a 26-year-old, gravida 4 mother. The prenatal course was complicated by maternal iron deficiency anemia and growth restriction for fetus FKE065. Amniotic fluid testing for each fetus revealed a normal 46,XX female karyotype. Breech presentation led to cesarean section delivery of small-for-gestational-age twins with Apgar scores ≥6 at 37-5/7 weeks gestation. Polymerase chain reaction (PCR)-based DNA zygosity testing of the twins and their mother indicated a 98.2% probability of monozygosity. Parents are of indigenous Ecuadorian ancestry with no known consanguinity.

Neonatal complications were significant for failed hearing screenings for both twins, and heart murmur and mild jaundice for FKE066 that resolved without phototherapy. On the seventh day of life when abnormal newborn screening results for galactosemia were reported (Table 1), exclusively soy formula feedings were initiated. On the 11th day of life, during the initial metabolic consultation (Table 1), the twins had normal physical examinations and normal alanine aminotransferase levels (Table 2). Diagnostic testing revealed impairment of GALE enzymatic activity in hemolysates in the affected range (Table 1). At 9 weeks of age during metabolic follow-up, the twins were found to have new onset jaundice, cholestasis, conjugated hyperbilirubinemia, elevated liver transaminases without coagulopathy, gastroesophageal reflux, and failure to thrive, prompting prolonged hospitalizations (Table 2).

Throughout their hospitalizations, failure to thrive persisted despite high-caloric soy formula intake and fat-soluble vitamin supplementation. Neither twin had hepatomegaly. Biliary atresia was discounted by radionuclide imaging. Renal findings in both twins included generalized amino aciduria with echogenic kidneys and

Table 1 Biochemical, enzymatic, and molecular testing results

	FKE065	FKE066	Reference Ranges
GALT enzyme activity ^a	12.7	13.4	Control: ≥ 3.2 units/g Hb
Total galactose level ^a	38.3	30.3	Control: ≤ 18.0 mg%
Galactose-1-phosphate ^b	$<0.1^c$, 0.2^c , 0.1^c , 0.8^d , 0.1^d , $<0.1^d$	0.2^c , 0.1^c , 0.1^c , 0.2^d , 0.4^d , 0.2^d , 0.5^d	Control: 0.0 – 1.0 mg%
Galactitol, urine	$22.6^{c,e}$, $19.0^{c,e}$, $32.7^{c,e}$	$29.9^{c,e}$, $26.0^{c,e}$, $38.6^{c,e}$	Control: 0 – 94.7 mmol/mol Cr
GALE enzyme activity ^b	5.8, 10.1	9.0, 7.9	Control: 17.1 – 40.1 μ mol/h/g Hb Carrier: 12 – 20 μ mol/h/g Hb Affected: 0 – 8 μ mol/h/g Hb (Shin 1991)
GALT enzyme activity ^b	28.7	26.9	Control: 22.2 – 45.8 μ mol/h/g Hb
Galactosemia isozymes	N/N	N/N	Control: N/N
GALK enzyme activity ^b	1.15	1.5	Control: 0.92 – 4.40 μ mol/h/g Hb
GALE gene sequencing	c.658C>T (p.R220W)/c.658C>T (p.R220W)	c.658C>T (p.R220W)/c.658C>T (p.R220W)	Control: no mutations detected

^a Blood spot newborn screen collected at 36 h of age^b Red blood cells^c Galactose-restricted diet (Gal-1P measured at <1 , 8, and 13 months for FKE065 and at <1 , 6, and 8 months for FKE066; galactitols collected twice at 2+ months and again at 3 months for each twin)^d Galactose-unrestricted diet (Gal-1P measured at 15, 21, and 34 months for FKE065 and 13, 15, 21, and 34 months for FKE066)^e Symptomatic cholestatic jaundice (9–16 weeks of age)**Table 2** Hepatic and endocrine laboratory results

	FKE065	FKE066	Normal reference range
Alanine aminotransferase	$4^{a,c}$, 44 – $215^{b,c}$, 47^d	$8^{a,c}$, 29 – $235^{b,c}$, 15^d , 26^e	0 – 50 u/L
Aspartate aminotransferase	79 – $435^{b,c}$, 66^d	62 – $399^{b,c}$, 76^d , 33^e	0 – 50 u/L
Alkaline phosphatase	683 – $1264^{b,c}$, 189^d	822 – $2285^{b,c}$, 190^d , 164^e	110 – 420 u/L
Bilirubin, conjugated	0.0 – $4.5^{b,c}$, 0.0^d	0.0 – $3.0^{b,c}$, 0.0^d	0 – 0.3 mg/dL
Bilirubin, total	1.3 – $7.6^{b,c}$, $<0.1^d$	0.7 – $5.9^{b,c}$, 0.6^d , 0.4^e	0.2 – 1.3 mg/dL
Bilirubin, delta	0.8 – $2.5^{b,c}$, 0.0^d	0.3 – $1.8^{b,c}$, 0.5^d	0.0 – 0.4 mg/dL
Gamma-glutamyltransferase	$520^{b,c}$	Not measured	0 – 130 u/L
Serum calcium	7.2 – $10.0^{b,c}$, 10.0^d	7.7 – $9.6^{b,c}$, 10.3^d	9 – 11 mg/dL
Ionized calcium	3.2 – $5.7^{b,c}$, 5.1^d	3.6 – $5.8^{b,c}$, 5.2^d	5.1 – 6.3 mg/dL
Serum phosphorus	1.1 – $4.9^{b,c}$, 5.0^d	1.6 – $3.9^{b,c}$, 5.9^d	3.9 – 6.5 mg/dL
Parathyroid hormone, intact	151 – $641^{b,c}$	175 – $929^{b,c}$	12 – 72 pg/mL
25-hydroxyvitamin D, total	<10 – $<36^{b,c}$, $<38^d$	<10 – $32^{b,c}$, $<34^d$	≥ 30 μ g/L

^a Asymptomatic, day 11 of age^b Acutely symptomatic 9–16 weeks of age^c Galactose-restricted diet (<1 –4 months of age)^d Obtained at age 34 months (after 16 consecutive months on galactose-unrestricted diet without calcium or vitamin D supplementation)^e Obtained at age 56 months (after 38 consecutive months on a galactose-unrestricted diet)

interstitial nephritis. They also had severe bone demineralization and subacute fractures leading to diagnoses of severe vitamin D–deficient rickets (Table 2). The rickets diagnoses were attributed to probable severe maternal vitamin D

deficiency, although maternal testing was unable to be completed. Osteogenesis imperfecta gene sequencing (COL1A1 and COL1A2) was later performed for FKE066 with negative results.

FKE066 had a cardiac murmur and mild supravulvar pulmonary stenosis. FKE065 also developed a cardiac murmur and was found to have supravulvar and branch pulmonary artery stenosis. Extensive infectious disease testing was negative for FKE066. FKE065 had high levels of cytomegalovirus (CMV) by DNA testing using PCR on multiple occasions, leading to liver biopsy at 12 weeks of age that suggested CMV hepatitis, and was treated with intravenous ganciclovir. Both infants had normal transcranial ultrasounds and normal ophthalmology examinations.

At hospital discharge, both infants showed healing rickets, correction of vitamin D deficiency after aggressive oral and IV therapy, resolution of cholestatic jaundice with ursodiol treatment and improvement of liver enzymes (Table 2).

In mid-infancy, both twins had high-resolution chromosome banding (46,XX), and array comparative genomic hybridization studies performed that demonstrated copy number losses consistent with deletions within band 7q11.23; FISH with a probe spanning the *LIMK* and *D7S613* loci confirmed the deletions and diagnoses of Williams syndrome. Subtle facial dysmorphism characteristic of Williams syndrome first became apparent for the infants at 8–11 months of age. By 16–21 months of age, both children had bitemporal narrowing, periorbital fullness, and full lips. FKE066 also had a coarse voice. By 5–8 months of age, the twins had hypotonia, brisk deep tendon reflexes, and language and gross motor delays. Neuropsychological evaluation at 10 months of age using the *Bayley Scales of Infant and Toddler Development* (Third Edition) documented pervasive developmental delays with cognitive, language, and motor age equivalencies in the 2–5 month range for both infants. Global developmental delays persisted; both twins sat unsupported at 13 months and walked independently at 28–29 months of age. At age 3 years, they remained largely nonverbal, hypotonic, and globally developmentally delayed.

GALE gene sequencing for each twin revealed apparent homozygosity for a previously unreported variant predicted by homology studies to be likely causative of GALE deficiency (Table 1). Their mother was later determined to be heterozygous for this sequence variant. Paternal genetic testing was unable to be performed. Galactose restriction was maintained throughout infancy as the GALE deficiency evaluation remained in progress, with galactose challenges undertaken at 13–15 months of age (Table 1). By 18 months of age, both children were on normal diets, unrestricted in galactose. Post-galactose challenge evaluations revealed normal eye examinations and no elevation of hemolysate Gal-1P for either twin (Table 1), and a normal liver ultrasound for FKE065.

At the time of this report, FKE065 has required 10 additional hospitalizations with subsequent bacteremia, gastrostomy tube placement (later removed), systemic

hypertension requiring antihypertensive therapy (resolved), acute renal insufficiency (resolved), and marked thoracic scoliosis. FKE066 has required two additional hospitalizations for respiratory infections. Both children have moderate bilateral sensorineural hearing loss. They have been discharged from further metabolic follow-up.

Results

hGALE and hGALT Activity in Patient Lymphoblast Cells

To characterize the ostensible GALE impairment in these twins, we prepared EBV-transformed lymphoblasts from each and tested the resulting cell lysates for GALT and GALE activities (Fridovich-Keil et al. 2011; Openo et al. 2006). Three control lines (FKT901, FKT934, and GM) and one from a patient previously diagnosed with intermediate epimerase deficiency galactosemia (FKE084) were analyzed in parallel.

Standard GALE assays of lysates from FKE065 and FKE066 demonstrated no significant impairment relative to the three unaffected controls (Fig. 1a, $P = 0.4671$) while the affected lysate, FKE084, demonstrated only about 21% of control activity ($P < 0.0001$). Of note, GALE activities detected in lymphoblasts from the twins (FKE065 and FKE066) differed by close to a factor of 2; this range was also observed in lymphoblasts from different unaffected controls (Fig. 1a; Openo et al. 2006). As expected, the GALT activity levels in all six cell lines were indistinguishable ($P = 0.1986$, Fig. 1b).

Characterization of Human GALE-R220W Expressed in Yeast

To assess the functional significance of the R220W substitution in a more controlled setting, we recreated the variation by site-directed mutagenesis and expressed the resultant allele from a low-copy (CEN) plasmid in a null-background strain of *S. cerevisiae*, JFy3835 (Chhay et al. 2008b). Parallel cultures of yeast expressing wild-type human GALE (WT) or no GALE (bb only) served as positive and negative controls, respectively (Wohlers et al. 1999; Quimby et al. 1997).

Under normal assay conditions (Chhay et al. 2008b) using either UDP-Gal or UDP-GalNAc as substrate, hGALE-R220W demonstrated >50% wild-type activity (Fig. 2a and 2b). However, the apparent $V_{\max}$ values for the wild-type and hGALE-R220W proteins differed by about two-fold when the substrate was UDP-Gal (Table 3), and the apparent K_m values differed by about a factor of 2 when the substrate was UDP-GalNAc (Table 4). These

Fig. 1 GALE and GALT enzyme activity levels in control and patient lymphoblast cells. EBV-transformed lymphoblast cells cultured in complete RPMI-1640 medium with 10% FBS were collected by centrifugation and soluble lysates were prepared. FKT901, FKT934, and GM were derived from non-galactosemic controls (white bars).

FKE065 and FKE066 represent the twins reported here (gray bars). FKE084 represents a patient with previously diagnosed intermediate epimerase deficiency (black bar). (a) GALE and (b) GALT enzyme activities were measured as described in Materials and Methods. Activity is expressed as mean $\pm$ SEM ($n \geq 4$) pmol/min/ μ g soluble protein

Fig. 2 Enzyme activity of R220W-hGALE expressed in null-background yeast. Colonies were inoculated from *GAL10*-null yeast transformed with plasmids encoding either wild-type hGALE (WT), no hGALE (backbone (bb) only), or R220W-hGALE (R220W). Cultures were expanded and harvested, and lysates prepared as

described in Materials and Methods. GALE enzyme activity was monitored using either (a) UDP-Gal or (b) UDP-GalNAc as substrate. Activity is expressed as mean $\pm$ SEM ($n \geq 4$) pmol/min/ μ g soluble protein

$V_{\max}$ and K_m differences might explain why the apparent activity of the hGALE-R220W-substituted protein was reduced by close to twofold relative to the wild-type protein when measured using the standard assay (Fig. 2).

We also tested for possible impact of the R220W substitution on cofactor dependence of the enzyme activity (Frey

1996; Thoden et al. 1996, 2001a) by monitoring reactions performed under initial conditions of fixed substrate concentration and varying amounts of exogenous NAD^+ . Although some patient mutations have been shown to disrupt hGALE interactions with NAD^+ (Quimby et al. 1997), we saw no evidence of such an impact by R220W (Fig. 3).

Table 3 Apparent K_m and V_{max} values of WT and R220W-hGALE proteins (UDP-Gal)

hGALE Derivative	Apparent UDP-Gal K_m (mean $\pm$ SEM mM)	Apparent UDP-Gal V_{max} (mean $\pm$ SEM pmol/min/ μ g protein)
WT	0.314 $\pm$ 0.014	456.69 $\pm$ 6.35
R220W	0.274 $\pm$ 0.023	212.58 $\pm$ 5.49*

Assays to determine the K_m and V_{max} of UDP-Gal were performed at the concentration of 0.5 mM NAD^+ . Kinetic constants were determined by fitting the data to a ligand-binding plot by SigmaPlot 11.0. All values are averages $\pm$ SEM ($n = 3$) of three independent analyses

*Indicates $P \leq 0.05$

Table 4 Apparent K_m and V_{max} values of WT and R220W-hGALE (UDP-GalNAc)

hGALE derivative	Apparent UDP-GalNAc K_m (mean $\pm$ SEM mM)	Apparent UDP-GalNAc V_{max} (mean $\pm$ SEM pmol/min/ μ g protein)
WT	0.165 $\pm$ 0.038	114.39 $\pm$ 7.24
R220W	0.339 $\pm$ 0.045*	111.62 $\pm$ 5.47

Assays to determining the K_m and V_{max} of UDP-GalNAc were performed at the concentration of 0.5 mM NAD^+ . Kinetic constants were determined by fitting the data to a ligand-binding plot by SigmaPlot 11.0. All values are averages $\pm$ SEM ($n = 3$) of three independent analyses

*Indicates $P \leq 0.05$

Galactose Sensitivity of Yeast Expressing hGALE-R220W

Finally, we measured the galactose-sensitivity of yeast expressing hGALE-R220W as their only GALE enzyme. Previously, we have demonstrated that the growth rate of yeast with compromised GALE activity is diminished in medium containing glycerol-ethanol as the carbon source when trace levels of galactose are spiked into the medium, and this effect is dose dependent (Ross et al. 2004; Wasilenko and Fridovich-Keil 2006). Here we tested the growth rates of yeast expressing wild-type hGALE (WT), no hGALE (bb only), or hGALE-R220W (R220W), each cultured in synthetic glycerol-ethanol medium without galactose vs. with 0.002% or 0.02% galactose added at $t = 0$ (Fig. 4). In medium lacking galactose, all three of the strains grew well (Fig. 4a), while in medium spiked with galactose, yeast expressing either wild-type hGALE or hGALE-R220W grew well, but yeast missing GALE did not (Fig. 4b, c). These data confirm that hGALE-R220W encodes a GALE enzyme that is predominantly, if not fully, functional in living yeast cells.

Fig. 3 Effect of NAD^+ on GALE activity detected in lysates of yeast expressing wild-type vs. R220W-hGALE. GALE assays were performed as described in Materials and Methods using soluble lysates of yeast expressing either wild-type hGALE or R220W-hGALE. (a) The initial concentration of substrate UDP-Gal was held constant at 0.8 mM; (b) the initial concentration of substrate UDP-GalNAc was held constant at 0.66 mM; NAD^+ concentration was varied as indicated. Activity is expressed as mean $\pm$ SEM ($n \geq 4$) pmol/min/ μ g soluble protein

Discussion

The purpose of this study was threefold. First, we wanted to illustrate the complex challenges of elucidating the clinical phenotype of epimerase deficiency in patients with concurrent comorbidities. In this case, outcome was confounded by the presence of additional genetic (Williams syndrome) and environmental (CMV and vitamin D deficiency) issues; had there not been a pair of monozygotic twins, both of whom shared the same GALE genotype, but who demonstrated discordant outcomes for some symptoms, it might have been tempting to attribute more of the clinical complications to compromised GALE function.

Second, we wanted to address the question: How does one distinguish a functionally neutral or near-neutral variant from a functionally significant GALE mutation, especially when the genetic background of the patient may be distinct from that of publicly available control populations? That the variant found in this family had not been reported in control populations (e.g., HapMap) of predominantly European, Asian, or African

Fig. 4 Yeast expressing R220W-hGALE demonstrate no apparent growth-sensitivity to galactose. Cultures of null-background yeast expressing either wild-type hGALE (WT), no hGALE (bb only), or R220W-hGALE (R220W) were inoculated into the wells of 96-well plates at a density of 5×10^5 cells/mL at $t = 0$ in SGE-Ura synthetic medium supplemented with the indicated amount of galactose. Growth of each culture was monitored as described in Materials and Methods. Values plotted represent means $\pm$ SEM ($n = 9$). (a) All cultures grew well in the absence of galactose. (b) Addition of 0.002% galactose halted the growth of yeast lacking hGALE but not yeast expressing WT or R220W-hGALE. (c) Addition of 0.02% galactose halted the growth of yeast lacking hGALE but not yeast expressing WT or R220W-hGALE

ancestry may not be particularly informative given that the family traces their roots to Ecuador. The situation described here was further complicated by the observation that the twins are apparently homozygous for this novel variant. Testing of the father was never performed and it remains possible that he is a carrier of a *GALE* allele, such as a deletion, that escapes detection by our assay and therefore results in apparent homozygosity of the other allele. If the twins are truly homozygous for the R220W variant allele, it might be common in the relevant ancestral population. Alternatively, there might be some unrecognized shared ancestry between the parents, leading to concern that the twins might be homozygous at other loci, as well, and these cryptic homozygosities might underlie at least some of the apparent clinical abnormalities. Of note, consanguinity was a

known confounding factor in both affected families of the first patients reported with generalized epimerase deficiency galactosemia (Henderson and Holton 1983; Holton et al. 1981; Sardharwalla et al. 1988; Walter et al. 1999) who demonstrated a variety of developmental or other disabilities. A patient reported more recently (Sarkar et al. 2010), who also presented with acutely symptomatic epimerase deficiency in infancy, responded well to dietary restriction of galactose and apparently has not exhibited developmental delays; it is unknown whether there is consanguinity in that family.

Finally, we wanted to address the balanced relationship between in silico predictions of functional significance of amino acid substitutions, predictions from in vitro studies of recombinant proteins, and indicators of function in vivo in a yeast model system. As discussed below, in silico, in vitro, and in vivo studies can give disparate results, leading to the need for a judgment call that balances the weight of the accumulated clinical and laboratory data.

Arguments pointing toward clinically significant epimerase deficiency: The initial newborn screening result of the twins showed elevated total galactose at about twice the normal cut-off (Table 2). Repeat follow-up hemolysate GALE enzyme assays showed reduced activity within the affected range (Table 1) and apparent homozygosity for a novel variant of GALE (R220W) that in silico prediction tools classified as likely to be of functional significance. Polyphen-2 predicted this amino acid replacement as “probably damaging” with a score 1.00. The arginine at position 220 in the GALE protein is conserved among numerous mammalian species. In addition, arginine is conserved at position 220 in the amphibian *Xenopus* as well as in various species of fish indicating that this amino acid is conserved through many different vertebrates. Indeed, only chicken had a different amino acid, glutamine, at this position.

Arguments pointing away from functionally significant epimerase deficiency galactosemia: The neonatal presentation of the twins was unremarkable despite the fact that both infants were on a milk diet, that the lymphoblast GALE activity assay showed essentially normal values, and that the yeast expression studies demonstrated only very marginally lowered GALE activity in vitro and no apparent GALE defect in vivo. It is important to note that acute jaundice and other complications did not present until months after the diet had been switched to exclusively soy formula (Case History). The marginal elevation of total galactose in the newborn screens, coupled with the intermediate and variable hemolysate GALE results reported for both twins from follow-up studies, leaves open the question of the functional significance of these data. Indeed, that the twins were delivered at less than 38 weeks gestation and small for gestational age suggests that “immature liver” might have contributed to the newborn

screening results (Fridovich-Keil et al. 2011; Ono et al. 1999). Finally, when taken off the galactose-restricted diet at 18 months, Gal-1P did not elevate in either twin, suggesting that the Leloir pathway in these infants was functioning, at least at that point.

Neutral variant or significant mutation? Novel missense variants at any genomic locus in a symptomatic patient are typically assessed for functional significance initially by two approaches: (1) querying prevalence of the variant in relevant affected and control populations and (2) in silico prediction programs that utilize multiple sequence alignments and structural information.

The twins reported here are apparently homozygous for an allele of GALE that has not been described previously; this suggests only that the allele may be uncommon in European and other studied populations. We have no information on the frequency of this allele among the indigenous peoples of Ecuador.

Due to the complex clinical picture of the twins (Williams syndrome and sensorineural hearing loss), additional steps were taken to test the functional significance of the identified missense variation; these included biochemical studies of transformed patient lymphoblasts, and in vitro and in vivo measures of GALE function in a null-background yeast strain engineered to express the relevant patient allele (R220W-hGALE). All of these measures demonstrated near-normal GALE activity, suggesting the R220W substitution does not significantly disrupt hGALE function, at least in non-peripheral cells. These results are consistent with a diagnosis of peripheral epimerase deficiency in FKE065 and FKE066. Some small differences (e.g., K_m or V_{max}) were detected in some assays between R220W-hGALE and the unaffected control; however, these differences were generally no more than a factor of 2, which is unlikely to impact clinical outcome in a recessive condition. Of course, we cannot rule out that the R220W substitution might have some significant but cryptic impact not tested for here, for example, restricted to a specific tissue, or time in development.

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One Sentence Summary

Detailed biochemical studies of a rare variant of human GALE, R220W, suggest that it is unlikely to account for the negative outcomes observed in the twins who carry it.

Contributions of the Individual Authors

Y Liu performed the majority of experiments presented, assembled the figures and tables, and wrote the first draft of the manuscript. K Bentler identified this family, assembled all of the clinical and some of the laboratory data, and wrote the manuscript section dealing with clinical presentation. B Coffee oversaw and interpreted the GALE genotyping and wrote the section of the manuscript relevant to those data. JS Chhay generated the hGALE-R220W yeast expression plasmid and also prepared and initially characterized the EBV-transformed lymphoblasts from both subjects. K Sarafoglou provided clinical consultation on endocrinology issues and assisted with writing of the manuscript section dealing with clinical presentation and some tables. JL Fridovich-Keil oversaw the project, wrote some sections of the manuscript, and edited and finalized all sections of the manuscript for submission. All authors assisted with editing the final manuscript.

Guarantor

Judith L. Fridovich-Keil is the guarantor for this work.

Competing Interest Statement

None of the authors has any competing interests to disclose.

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Ethics Approval

This project was conducted with approval from the Emory University Institutional Review Board (Protocol # 618–99, PI: JL Fridovich-Keil) and study volunteers were consented prior to the study in accordance with that protocol.

No vertebrate animals were used in this study.

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