Biallelic *GALM* pathogenic variants cause a novel type of galactosemia

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Purpose: Galactosemia is caused by metabolic disturbances at various stages of galactose metabolism, including deficiencies in enzymes involved in the Leloir pathway (GALT, GALK1, and GALE). Nevertheless, the etiology of galactosemia has not been identified in a subset of patients. This study aimed to explore the causes of unexplained galactosemia.

Methods: Trio-based exome sequencing and/or Sanger sequencing was performed in eight patients with unexplained congenital galactosemia. In vitro enzymatic assays and immunoblot assays were performed to confirm the pathogenicity of the variants.

Results: The highest blood galactose levels observed in each patient were 17.3–41.9 mg/dl. Bilateral cataracts were observed in two patients. In all eight patients, we identified biallelic variants (p.Arg82*, p.Ile99Leufs*46, p.Gly142Arg, p.Arg267Gly, and p.Trp311*) in the *GALM* encoding galactose mutarotase, which

catalyzes epimerization between β - and α -D-galactose in the first step of the Leloir pathway. *GALM* enzyme activities were undetectable in lymphoblastoid cell lines established from two patients. Immunoblot analysis showed the absence of the *GALM* protein in the patients' peripheral blood mononuclear cells. In vitro *GALM* expression and protein stability assays revealed altered stabilities of the variant *GALM* proteins.

Conclusion: Biallelic *GALM* pathogenic variants cause galactosemia, suggesting the existence of type IV galactosemia.

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INTRODUCTION

Lactose accounts for almost all sugar present in breast milk or formula and is a major source of energy for newborns and infants. Lactose is cleaved into D-glucose and β -D-galactose on the intestinal villi by β -D-galactosidase. β -D-Galactose is taken up into the liver via the portal vein and primarily metabolized by the Leloir pathway, which is highly conserved and consists of four individual enzymes: galactose mutarotase (or aldose 1-epimerase) (*GALM*, EC 5.1.3.3), galactokinase (*GALK1*, EC 2.7.1.6), galactose-1-phosphate uridylyltransferase (*GALT*, EC 2.7.7.12), and UDP-galactose-4'-epimerase

(*GALE*, EC 5.1.3.2) (Supplementary Figure S1) (refs. ^{1–3}). Galactosemia is one of the disorders detected in newborn screens⁴ and is caused by disturbances in various steps of galactose metabolism, including deficiencies in the enzymes in the Leloir pathway, portosystemic shunts, cholestasis (e.g., citrin deficiency [MIM 605814] [refs. ^{5,6}] and neonatal hepatitis), and other metabolic disorders, such as Fanconi–Bickel syndrome (MIM 227810). The first step of the Leloir pathway involves epimerization between β - and α -D-galactose, which is catalyzed by *GALM*.⁷ The next three genes in the Leloir pathway (*GALT* [MIM 606999] [refs. ^{8,9}],

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GALK1 [MIM 604313] [ref. ¹⁰], and *GALE* [MIM 606953] [refs. ^{11–13}]) have been identified as being responsible for type I, II, and III galactosemia (*GALT* [MIM 230400], *GALK1* [MIM 230200], and *GALE* [MIM 230350] deficiencies), respectively.^{1,7}

In patients with galactosemia, appropriate care and precise diagnosis are essential to avoid complications. Unless breast milk or normal formula is substituted with galactose/lactose-free formula, cataracts often develop. Severe symptoms, including a failure to thrive, gastrointestinal symptoms, liver dysfunction, sepsis, and death, may occur in patients with classic galactosemia (severe *GALT* deficiency)² or patients with a generalized *GALE* deficiency.^{14,15} In adolescent and adult patients with a *GALT* deficiency, long-term complications, such as neuropsychological symptoms and premature ovarian failure, have also been observed.¹⁶ Surgical intervention may be required in certain patients with portosystemic shunts. Thus, a differential diagnosis of the type of galactosemia is crucial for appropriate care. Nevertheless, the etiology of some galactosemia cases remains unknown.¹⁷ We encountered a group of patients with unexplained and persistent galactosemia who had did not present with pathogenic variants in *GALT*, *GALK1*, or *GALE*, portosystemic shunts, cholestasis, or no urinary glucose, which is commonly present in patients with Fanconi–Bickel syndrome.

We performed exome sequencing in these patients with unexplained galactosemia to explore the cause of this condition, and identified biallelic pathogenic variants in *GALM* (MIM 137030) for the first time. We describe the genetic and clinical features of a novel type of galactosemia associated with *GALM* deficiency in the present study.

MATERIALS AND METHODS

Patients

We studied eight Japanese patients with unexplained congenital galactosemia (Table 1, Supplemental Note). Initially, we studied two unrelated patients with galactosemia (patients 1 and 2) whose clinical data were not consistent with *GALT*, *GALK1*, and *GALE* deficiencies, portosystemic shunt, cholestatic diseases, or Fanconi–Bickel syndrome. Urine glucose test results were negative in these two patients, excluding a diagnosis of Fanconi–Bickel syndrome. The Beutler test was negative, excluding the possibility of a *GALT* deficiency. Detection of blood galactose-1-phosphate (Gal-1-P) (cut-off value: 15 mg/dl) and elevated galactose levels (cut-off value: 3 mg/dl) in the two patients in the newborn screen suggested the possibility of a *GALE* deficiency rather than a *GALK1* deficiency; however, their *GALE* activities in red blood cells were normal (Table 1). Variants in *GALT*, *GALK1*, and *GALE* were screened in the two patients, but no pathogenic variants were identified.

Six additional patients with unexplained congenital galactosemia were enrolled. Their clinical data were not consistent with *GALT*, *GALK1*, or *GALE* deficiencies, or Fanconi–Bickel syndrome (Table 1). Patients 5 and 8 received a diagnosis of galactosemia caused by a portosystemic shunt, but their

galactosemia persisted after spontaneous closure of the shunts. No pathogenic variants in *GALT*, *GALK1*, and *GALE* were identified in these six patients, with the exception of a heterozygous variant, c.446C>T, p.(Thr149Met), in *GALK1* identified in patient 3. Although this variant was rare and predicted to affect protein function by certain prediction tools, the amino acid sequence is not conserved among vertebrates. In particular, rhesus monkeys express a methionine, the same residue as the variant allele of patient 3, in this position. In addition, we did not identify other candidate variants in the same gene. Therefore, we did not pursue this variant further.

The present study was approved by the ethics committee of Tohoku University School of Medicine. Written informed consent was obtained from the patients' parents.

Sequencing analysis

For patients 1 and 2, we performed trio-based exome sequencing. Genomic DNA was captured using SureSelectXT Human All Exon V6 (60 Mb) Kits (Agilent Technologies, Santa Clara, CA, USA) and sequenced on a HiSeq2500 (Illumina, San Diego, CA, USA) with 126-base pair paired-end reads. The reads were mapped to the hg19 human reference genome using Novoalign v. 2.08.05 (<http://www.novocraft.com>), and single-nucleotide variants (SNVs) and insertions and/or deletions (indels) were identified using the Genome Analysis Toolkit (GATK) v. 1.6-13 (ref. ¹⁸). After quality filtering steps, variants were annotated using ANNOVAR.¹⁹ Nonsynonymous SNVs, splice-site variants, and indels were retained. Because both sexes were affected, we hypothesized an autosomal recessive mode of inheritance. We excluded variants present at <1% minor allele frequency (MAF) in the 1000 Genomes database, the ExAC database version 0.3 (all and East Asian populations), and the Human Genetic Variation Database (HGVD).^{20,21} For patients 3–8, Sanger sequencing of all exons and flanking regions of *GALM* was performed.

In vitro expression analysis

The *GALM* complementary DNA (cDNA) (IRAK015M11) was provided by the RIKEN Bioresource Center (BRC) through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan,^{22–25} and was subcloned into pCMV6-AN-DDK (PS100014, OriGene, Rockville, MD, USA) or pET21d(+) (69743, Novagen, Merck Millipore, Darmstadt, Germany) with a His6 tag at the N-terminus using the In-Fusion HD Cloning Kit (TaKaRa, Shiga, Japan). Then, 293FT cells (R700-07, Invitrogen™) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing high glucose supplemented with 10% fetal bovine serum, and 1% penicillin and streptomycin (100 U/ml and 100 µg/ml, respectively) at 37 °C in a 5% CO₂ incubator. Epstein–Barr virus transformed lymphoblastoid cell lines (EBV-LCLs) were established from patients 2, 3, 8, and healthy controls using previously described methods.²⁶ All cell lines were tested for mycoplasma

Table 1 Clinical and genetic characteristics of patients with GALM pathogenic variants

Patient		1	2	3	4	5	6	7	8
General information									
GALM variant		c.[244C>T]; [294delC] p.[Arg82*]; [Ile99Leufs*46]	c.[294delC]; [799C>G] p.[Ile99Leufs*46]; [Arg267Gly]	c.[294delC];[294delC] p.[Ile99Leufs*46]; [Ile99Leufs*46]	c.[932G>A]; [932G>A] p.(Trp311*); (Trp311*)	c.[424G>A]; [(424G>A)] p.(Gly142Arg)(:); (Gly142Arg)	c.[424G>A]; [(424G>A)] p.(Gly142Arg)(:); (Arg267Gly)	c.424G>A(:) 799C>G p.(Gly142Arg)(:) (Arg267Gly)	c.[424G>A]; [424G>A] p.[Gly142Arg]; [Gly142Arg]
Sex		Boy	Girl	Boy	Girl	Boy	Girl	Boy	Boy
Consanguinity		-	-	-	-	-	-	-	-
Gestational age, wks		40	38	39	37	37	40	38	40
Birth weight, g		3414	2914	3424	2132	2946	2802	2696	3118
Perinatal adverse events		-	-	-	-	Neonatal jaundice	-	-	-
Maximum galactose level, mg/dl (age)		17.3 (44 d)	41.9 (4 m)	19.2 (16 d)	28.2 (5 m)	29.8 (8 m)	34.0 (12 d)	31.0 (6 m)	34.1 (13 d)
Galactose and Gal-1-P levels at newborn screening									
Galactose, mg/dl		12.4	8.7	11.9	9.7	10.0	12.8	15.7	11.4
Gal-1-P, mg/dl		8.9	8.9	6.6	2.4	10.8	0.3	10.4	6.7
Gal-1-P/galactose ratio		0.7	1.0	0.6	0.2	1.1	0.0	0.7	0.6
Activity of enzymes involved in galactose metabolism									
Beutler test		Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
GALE activity		Normal	Normal	Normal	NA	NA	Normal	Normal	NA
Clinical Features									
Cataracts		+	-	-	-	-	-	-	+
Hepatomegaly		-	-	-	-	-	-	-	-
Cholestasis		-	-	Transient	-	-	-	Transient	-
Poor weight gain		-	-	-	-	-	-	-	-
Short stature		-	-	-	-	-	-	-	-
Neurological symptoms		-	-	-	-	-	-	-	-
Portosystemic shunt		-	-	-	-	Spontaneous regression	-	-	Spontaneous regression
Galactose-free diet		Continuing	Continuing	Continuing	Discontinued	Continuing	Continuing	Discontinued	Continuing

d days, g grams, Gal-1-P galactose-1-phosphate, GALE UDP-galactose-4-epimerase, GALM galactose mutarotase, m months NA not available, wks weeks, + present, - absent.

contamination using a MycoAlert™ Mycoplasma Detection Kit (LT07-118, Lonza, Basel, Switzerland).

Cycloheximide protein stability assay

The pCMV6-FLAG-GALM (wild-type, G142R, R267G, W311*) plasmid was transfected into 293FT cells using Lipofectamine LTX (15338100, Thermo Fisher Scientific Inc., MA). Twenty-four hours after transfection, cells were treated with 20 µg/ml of cycloheximide. Lysates were harvested 0, 1, 4, or 12 hours after treatment and subjected to an immunoblot analysis, as described below.

Immunoblot analysis

We performed immunoblot assays on proteins extracted from peripheral blood mononuclear cells (PBMCs) or 293FT cells expressing FLAG-GALM. Antibodies used in the present study were anti-FLAG (F3165, Sigma-Aldrich, Merck Millipore), anti-GALM (16022-1-AP, Proteintech, Rosemont, IL, USA), peroxidase-conjugated anti-β-Actin (017-24573, Wako, Osaka, Japan), HRP-conjugated anti-mouse IgG (NA9310V, GE Healthcare UK Ltd, Buckinghamshire, England), and HRP-conjugated antirabbit IgG (NA9340V, GE Healthcare UK Ltd).

Analysis of GALM enzyme activity in EBV-LCLs

GALM enzyme activity was measured in EBV-LCLs obtained from the patients using a previously described coupled assay,^{7,27,28} with minor modifications. GALM catalyzes the conversion of α-D-galactose to β-D-galactose, followed by the reduction of NAD⁺ to NADH coupled with the reaction of β-D-galactose to β-galactonic acid catalyzed by β-galactose dehydrogenase. This reaction was performed in a solution containing 360 µM freshly dissolved D(+)-galactose (071-00032, Wako); 3 mM NAD⁺; 2.5 U β-galactose dehydrogenase; 10 mM HEPES, pH 7.4; and approximately 14 µg of EBV-LCL lysate. We confirmed that the D(+)-galactose almost completely consisted of α-D-galactose using nuclear magnetic resonance (NMR) (Supplementary Figure S2). The rate of increase in the absorbance of NADH at 339 nm was monitored using a SPECTRA MAX 190 instrument (Molecular Devices, CA, USA) immediately after the dissolution of α-D-galactose. These rates were corrected by subtracting the absorbance measured in the absence of cell lysates. We were not able to measure the GALM activity using PBMCs because control PBMCs exhibit very low enzymatic activity.

Statistical analysis

The statistical analysis was performed with JMP Pro 13.1.0. *P* values <0.05 were considered statistically significant.

RESULTS

Identification of pathogenic variants in GALM and the spectrum of pathogenic variants

Initially, we performed trio-based exome sequencing on two unrelated patients (patients 1 and 2). The mean depth of each exome was approximately 100. Trio-based exome sequencing

revealed only one candidate gene, *GALM* (NM_138801.2), for an autosomal recessive model (Supplementary Table S1). No rare de novo variants shared by the two probands were identified. An additional Sanger sequencing analysis identified six patients with unexplained congenital galactosemia and biallelic variants in *GALM*. Three patients (patients 1, 3, and 4) were homozygous or compound heterozygous for stop-gain variants or a frameshift variant leading to a premature termination in *GALM*; three patients (patients 5, 6, and 8) were homozygous for a missense variant, p.Gly142Arg; one patient (patient 7) was compound heterozygous for different missense variants; and one patient (patient 2) was compound heterozygous for missense and nonsense *GALM* variants (Table 1). Collectively, we identified five different *GALM* pathogenic variants (three stop-gain and two missense) in eight patients from unrelated families of Japanese descent (Table 1, Supplementary Table S2). All variants identified in the *GALM* gene are rare (<0.1% MAF), and no homozygous carriers of these variants were identified among 60,706 exomes in ExAC or 1208 people of Japanese ancestry in HGVD, whereas only one homozygous carrier of c.932G>A, p.Gly142Arg from Africa, was identified in gnomAD (Supplementary Table S2). Moreover, no homozygous stop-gain or frameshift genotypes in *GALM* were found in these databases. Approximately half (7/16) of the variants were p. Gly142Arg. According to the multiple sequence alignment, two missense variants, p.Gly142Arg and p.Arg267Gly, affect evolutionarily conserved residues (Supplementary Figure S3). The two missense variants were also predicted to be pathogenic by different in silico prediction tools (SIFT, PolyPhen-2, and MutationTaster) (Supplementary Table S2).

Measurement of GALM enzyme activity in EBV-LCLs using a coupled assay

We measured the mutarotase activity using a coupled assay. EBV-LCLs were available from two patients (patients 2 and 3). Patients' EBV-LCLs were unable to convert α-D-galactose to β-D-galactose, but the rate of α-D-galactose conversion in three control samples was greater than a buffer control (Fig. 1).

The GALM protein was not expressed in patients' PBMCs

PBMCs were available from three patients (patients 2, 3, and 8) carrying biallelic *GALM* variants. An immunoblot analysis with an anti-GALM antibody revealed the lack of the GALM protein in PBMCs from all three patients compared with control cells (Fig. 2a). Among the three patients, patients 2 and 8 harbored two missense variants and stop-gain or frameshift variants in *GALM* (p.[Ile99Leufs*46];[Arg267Gly] and p.[Gly142Arg];[Gly142Arg]), respectively. Based on this result, the stability of the variant GALM proteins is affected in cells harboring the missense variants.

Exogenous variant GALM expression was reduced in 293FT cells

We overexpressed the wild-type or variant FLAG-tagged GALM protein in 293FT cells to evaluate the possibility that

Fig. 1 Relative GALM enzyme activities in peripheral blood mononuclear cells (PBMCs), as determined using coupled assays. Individual data and means of three independent experiments are shown. The average of three controls in each experiment was set to 1. AU arbitrary units. * $P = 0.03$, ** $P = 0.02$, *** $P < 0.001$ (Tukey–Kramer test).

the pathogenic variants identified in patients affect the stability of the GALM protein. Among the variants, R82* and 199Lfs variants produced no or low levels of truncated protein, and other variants, G142R, R267G, and W311*, showed compromised expression of GALM (Fig. 2b).

Pathogenic variants in *GALM* affect the stability of GALM proteins

The compromised expression of the three variants prompted us to evaluate whether these variant GALM proteins exhibit a shorter half-life. According to the results of the cycloheximide protein stability assays, G142R, R267G, and W311* variants markedly reduced protein stability, as the variant proteins displayed shorter half-lives than the wild-type protein (Fig. 2c).

Recombinant variant GALM proteins yielded approximately 50% less protein upon expression in *E. coli* than the wild-type protein

We prepared recombinant GALM proteins to determine the specific activity of the GALM missense variants. Although the yield of the wild-type protein was approximately 5 mg of GALM protein per liter of bacterial culture, the respective yields of the two missense constructs (G142R, R267G) were approximately 1% and 4% of the yield of the wild-type GALM protein. This reduction occurred because most of the variant proteins were present in the insoluble fraction (data not shown), suggesting that the two missense pathogenic variants

affect protein folding. The enzymatic activity of the recombinant proteins carrying the two missense variants was approximately half of the activity of the wild-type protein (Supplementary Figure S4). This outcome is in line with the fact that the two residues (G142 and R267) are far from the active site that is involved in sugar binding²⁹ (Supplementary Figure S5). Thus, the two missense variants affect the quantity, but not the quality, of GALM proteins, consistent with the results of the immunoblot analysis of PBMCs.

The phenotypic spectrum of patients carrying *GALM* pathogenic variants

The highest blood galactose levels observed in each patient carrying *GALM* pathogenic variants were 17.3–41.9 mg/dl (Table 1, Supplemental Note). The Gal-1-P levels were highest in the neonatal period and decreased below 1 mg/dl in early infancy in each patient (Table 1, Supplemental Note, Fig. 3). None of the eight patients presented with gastrointestinal symptoms or severe liver dysfunction, which are caused by a GALT deficiency or generalized GALE deficiency. Two patients (patients 1 and 8) presented with bilateral cataracts. The presence of long-term complications, such as neuropsychological impairments or premature ovarian failure, was not clear because no adult patients were examined in the present study, although we do not have these complications to date. All patients were treated with a galactose-restricted diet immediately after the diagnosis of galactosemia, and therefore the natural history of GALM deficiency remained unknown. Two of these patients (patients 4 and 7) were allowed to resume a normal diet.

By comparing Gal-1-P/galactose ratios in newborn screens obtained from patients with a GALM deficiency, GALE deficiency, and portosystemic shunt using in-house data, we were able to differentiate between GALM deficiency and GALE deficiency. The ratios observed in patients with a GALM deficiency were similar to the ratios observed in patients with a portosystemic shunt (Fig. 4). Two patients (patients 5 and 8) carrying *GALM* pathogenic variants also had portosystemic shunts.

DISCUSSION

We identified biallelic pathogenic variants in *GALM* in eight patients with unexplained galactosemia, suggesting the presence of a novel type of galactosemia, namely, type IV galactosemia. Enzymatic activities of GALM were abolished in EBV-LCLs from patients 2 and 3 (Fig. 1). Immunoreactive GALM protein was undetectable in PBMCs from patients 2, 3, and 8 (Fig. 2a). In vitro GALM expression and protein stability assays revealed alterations in the stability of variant GALM proteins. According to the results of the recombinant protein analysis, the two missense pathogenic variants (p. Gly142Arg and p. Arg267Gly) affect the quantity but not the quality of the protein (Fig. 2, Supplementary Figure S4). Thus, the novel type of galactosemia identified in the present study is caused by loss-of-function variants in *GALM*, which is the only gene that has never previously been associated with

Fig. 2 Alterations in the stability of variant GALM proteins. **a** GALM expression in peripheral blood mononuclear cells (PBMcs), as determined by immunoblotting. β -Actin was used as a loading control. Representative results from three independent experiments are shown. **b** Immunoblot analysis of GALM expression. Transiently expressed FLAG-GALM was detected with an anti-FLAG antibody. β -Actin was used as a loading control. The expected position of a truncated protein product stemming from the I99Lfs allele is indicated with the arrowhead. Representative results from three independent experiments are shown. **c** Cycloheximide protein stability assay. Times at which cells were harvested after treatment are indicated. β -Actin was used as a loading control. Representative results from three independent experiments are shown. WT wild type.

galactosemia among the four genes encoding enzymes involved in the Leloir pathway.

GALM deficiency leads to persistent galactosemia, which should be treated appropriately. In this study, when patients with GALM deficiency consumed normal formula or were breast fed transiently before the weaning period, their blood galactose levels were elevated (Table 1, Fig. 3) as high as 41.9 mg/dl (Supplemental Note). While nonenzymatic conversion of β -D-galactose to α -D-galactose occurs in water,³⁰ galactose mutarotase is necessary for normal cell growth at least in some organisms, including yeast and *Escherichia coli*, probably because the rate of nonenzymatic conversion is slower in those cells than in water.^{31,32} It is likely that mammals, including humans, similar to yeast and *Escherichia coli*, also need galactose mutarotase for the efficient anomerization of galactose, particularly during the lactation period, when they consume large quantities of lactose. In addition, bilateral congenital cataracts were observed in two patients (patients 1 and 8). In patient 1, the cataracts were resolved after the start of a galactose-restricted diet (Supplemental Note). This finding indicates that the cataracts were likely caused by galactosemia due to the GALM

deficiency. Thus, patients with a GALM deficiency should be diagnosed and treated correctly, although severe complications, such as neurological symptoms, have not developed to date.

The phenotypes of GALM deficiency are similar to those of GALK1 deficiency, which abolishes the next step in the Leloir pathway (Supplementary Figure S1), as the only consistent clinical symptom of GALK1 deficiency is also cataracts.^{33,34} GALM deficiency shows more severe phenotypes than “peripheral” GALE deficiency, which causes virtually no clinical abnormalities except galactosemia.³⁵ Because all of the patients enrolled in the present study were treated with a galactose-restricted diet immediately after the diagnosis of galactosemia, the natural history of patients with a GALM deficiency, as well as the long-term prognosis, remain unknown. Although a nonsynonymous polymorphism in GALM has been associated with serotonin transporter binding potential in the human thalamus in a genome-wide association study,³⁶ the phenotypic significance of this association remains to be identified. Elucidation of the natural history and long-term prognosis is essential for clinicians to provide an accurate diagnosis of GALM deficiency.

Fig. 3 Time courses of galactose and Gal-1-P levels and the amount of galactose intake in patient 1. The galactose intake is a ratio of the intake of galactose-containing milk (normal formula) to the total milk intake (upper). The blood galactose level was elevated in response to a relaxing of galactose restriction (lower). Gal-1-P galactose-1-phosphate, AU arbitrary units.

Heterozygous carriers of a pathologic *GALM* variant were identified in public variant databases derived from populations of various ethnicities, suggesting that *GALM* deficiency exists in many countries. According to gnomAD, a predicted loss-of-function variant, p.Gly212*, was detected in 72 of 25,514 alleles in the Finnish population. Another predicted loss-of-function variant, p.Glu329Argfs*18, was present in 38 of 33,420 alleles in the Latino population. The p.Gly142Arg variant was identified in 7 of 16 Japanese variant alleles as a loss-of-function variant (Fig. 2). The pathogenic variant was also observed in 152 of 24,026 alleles in the African population. Interestingly, there is one African individual who is homozygous for p.Gly142Arg out of 277,212 alleles in the gnomAD database (Supplementary Table S2). The severity of the disease in homozygotes may be highly variable, particularly in patients with different ethnicities. Particularly in countries that perform a neonatal screen for galactosemia, it is also likely that patients with galactosemia caused by a *GALM* deficiency received a galactose-restricted diet due to the unexplained galactosemia but were allowed to resume a normal diet after the weaning period without any sequelae such as cataracts, as with our patients 4 and 7. The restriction for patients with unexplained galactosemia might have prevented us from making the correct diagnosis of *GALM* deficiency. A carrier frequency analysis would be helpful for evaluating the prevalence of galactosemia caused by *GALM* deficiency in those countries.

The differentiation between *GALM* deficiency and portosystemic shunt exclusively based on biochemical and imaging analyses is sometimes difficult. We compared galactose and

Fig. 4 Comparison of the Gal-1-P/galactose ratios in a newborn screen from patients with a *GALM* deficiency, *GALE* deficiency, and portosystemic shunt. In-house data from patients with a *GALE* deficiency and portosystemic shunts were used. * $P = 0.81$, ** $P < 0.001$ (Steel–Dwass test).

Gal-1-P levels in newborn screens between patients with a *GALM* deficiency, *GALE* deficiency, and portosystemic shunts. Gal-1-P/galactose ratios in patients with a *GALM* deficiency were similar to patients with portosystemic shunts, whereas the ratios were significantly elevated in patients with a *GALE* deficiency (Fig. 4). Notably, patients 5 and 8 were diagnosed with portosystemic shunts based on the presence of high levels of total bile acids and imaging findings at the initial diagnosis of galactosemia. *GALM* deficiency should be considered when galactosemia persists, despite the disappearance of sonographic findings of a portosystemic shunt or decreased levels of total bile acids. A proposed flow chart for the diagnosis of congenital galactosemia is shown in Supplementary Figure S6.

The mechanism of Gal-1-P fluctuations in patients with *GALM* deficiency remains to be elucidated. In the neonatal period, the Gal-1-P levels were relatively elevated, while the Gal-1-P levels declined in the postneonatal period (Table 1, Fig. 3, Supplemental Note). The profiles of galactose and Gal-1-P in the postneonatal period are similar to those associated with *GALK1* deficiency. A possible explanation for the relatively high Gal-1-P levels in neonatal patients with *GALM* deficiency is that that galactokinase activities in neonates are higher than those in infants and adults.³⁷ Future research should address whether these

fluctuations in Gal-1-P are unique to patients with GALM deficiency.

The detection of accumulated β -D-galactose, the substrate of GALM, might enable clinicians to biochemically diagnose GALM deficiency, in addition to a genetic diagnosis. We have established separate detection systems for α - and β -D-galactose using NMR (Supplementary Figure S2), and examined the anomers of galactose extracted from blood or dried blood spots on Guthrie cards obtained from control individuals and patients with a GALM deficiency. No significant differences in the galactose anomer profiles were observed between the controls and the patients (data not shown), probably because anomerization from one galactose anomer to the other occurs spontaneously and rapidly in water,³⁰ resulting in a complete equilibrium between α - and β -D-galactose (Supplementary Figure S2). Further studies are necessary to establish chemical methods for diagnosing GALM deficiency.

In summary, to the best of our knowledge, this study is the first to report an association between biallelic pathogenic variants in *GALM* and a novel type of galactosemia (type IV galactosemia). We recommend that an analysis of *GALM* variants should be added to the diagnostic workup of patients with unexplained galactosemia.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (<https://doi.org/10.1038/s41436-018-0340-x>) contains supplementary material, which is available to authorized users.

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DISCLOSURE

The authors declare no conflicts of interest.

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