

The molecular basis of UDP-galactose-4-epimerase (GALE) deficiency galactosemia in Korean patients

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Purpose: UDP-galactose-4-epimerase (GALE) deficiency galactosemia is an autosomal recessive disorder and the prevalence of the disease varies among ethnic groups. We aimed to investigate molecular characteristics of the Korean patients with attenuated GALE activity and elevated galactose-1-phosphate levels in blood. **Methods:** In order to characterize the molecular defects underlying GALE deficiency, the GALE gene of 7 patients showing severe activity decreases was sequenced. PCR-RFLP was performed to confirm the presence of the mutations identified by sequencing. **Results:** Nine mutations were identified: 8 missense mutations (p.A25V, p.R40C, p.D69E, p.E165K, p.R169W, p.R239W, p.G302D, and p.R335H) and one nonsense mutation (p.W336X). Except for p.R335H, all of these mutations are novel. Six patients were compound heterozygotes (p.D69E/p.G302D, p.R40C/p.R169W, p.D69E/p.E165K, p.R239W/p.R335H, p.A25V/p.R169W, and p.G302D/p.R335H) and the remaining patient had only one mutation (p.W336X/not detected). Thirty patients with moderately reduced GALE activity were also tested by PCR-RFLP for the presence of the above mutation, and mutations were detected in 17 of these 30 patients. The frequency of p.G302D (9/30), p.R239W (6/30) and p.R169W (5/30) in our Korean patients with GALE deficiency galactosemia was relatively high. **Conclusions:** We detected 9 mutations of the GALE gene in Korean galactosemia patients, and confirmed allelic heterogeneity in this disease. **Genet Med 2005;7(9): 646–649.**

Key Words: galactosemia, UDP-galactose-4-epimerase (GALE), mutation, Korean

INTRODUCTION

UDP-galactose-4-epimerase (GALE: EC 5.1.3.2) deficiency galactosemia (MIM# 230350) is an autosomal recessive disorder and is diagnosed in newborn screening by an increase in galactose or galactose-1-phosphate levels and a decrease in GALE enzyme activity. The impairment of GALE in humans results in one of two clinically distinct forms of epimerase deficiency galactosemia: one benign or peripheral,^{1–3} the other severe or generalized.^{4–8} In its benign or peripheral form,¹ epimerase deficiency is limited to red blood cells and uncultured white blood cells, and epimerase activities in liver, cultured skin fibroblasts, and activated lymphocytes are normal. This benign form of GALE deficiency appears to be relatively common among Japanese and black people compared to

nonblacks.^{2,9} In the generalized form, two patients, one Pakistani and the other Asian Muslim, have been described as presenting clinical symptoms in the newborn period similar to classical galactosemia including vomiting, jaundice, and hepatomegaly.^{4,5} Epimerase is deficient in all tissue types. A galactose restriction diet helpfully reduces acute symptoms, but long-term complications are not preventable. Recently, a unique patient with an intermediate GALE-deficiency phenotype has been reported showing impaired neurological function but none of the symptoms of acute toxicity.⁹

Since the human GALE gene was first cloned,¹⁰ a total of nine missense mutations have been identified in patients with GALE deficiency, including p.G90E, p.D103G, p.K257R, p.L313M, p.G319E,³ p.V94M,⁷ p.N34S, p.L183P,¹¹ and p.R335H.¹²

Biochemical studies show three of these mutations (p.G90E, p.V94M, and p.L183P) produce proteins with defective enzyme activity. Even though a p.V94M mutation was reported to be associated with the generalized form of epimerase deficiency,^{7,8,13} the genotype/phenotype relationships of the other mutations are unclear.

In this study we identified 37 newborns with peripheral GALE deficiency. Molecular analysis of the GALE gene in patients with severe defects in GALE activity identified nine mutations including eight missense and one nonsense.

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MATERIALS AND METHODS

Patients information

The research was approved by the Seoul National University Bundang Hospital institutional review board and written informed consents were obtained from the subjects and/or their parents. Korean patients with galactosemia were selected from the neonatal screening results for the past few years. They were consulted to Seoul National University Hospital for the enzyme assays of GALK, GALT, and GALE. Measurement of three enzyme activities was carried out in red blood cells by radiometric assay as described elsewhere.¹⁴

DNA amplification and direct sequencing

Human genomic DNA was isolated from frozen white blood cells using a QIAamp DNA Blood Mini Kit[®] (Qiagen, Germany) or a LaboPass[™] Blood Mini kit (Cosmogenetech, Seoul, Korea). Polymerase chain reaction was performed on genomic DNA to amplify all coding exons and flanking intron-exon junctions of the GALE gene using a set of 10 primer pairs as described elsewhere.³ PCR products were purified using a QIAquick PCR Purification Kit[®] (Qiagen, Germany) or QIAquick Gel Extraction Kit[®] (Qiagen, Germany), and sequenced using an ABI Prism[®] BigDye[™] Terminator Cycle Sequencing Ready Reaction Kit v3.1 protocol. Data were analyzed using an ABI3730XL DNA sequencer (Applied Biosystems, USA).

Confirmation of mutations in GALE by PCR-RFLP

In order to confirm the presence of the mutations identified by sequencing, PCR products were digested with the restriction enzymes detailed below. Primer pairs used were the same as those used for sequencing except that a mismatched reverse primer of exon 2 (reverse primer: 5'-TGACCACAGGCAAG-TAGCTA-3') was used to create a new cleavage site. Restriction enzymes used to confirm each mutation are *BfaI*, *BtgI*,

Sau96I, *TaqI*, *HphI*, *HaeII*, *NheI* (NEB, USA), and *HpaII* (KO-SCO, Korea) as shown in Table 1.

RESULTS

We identified 37 Korean patients with reduced GALE activity and an increased galactose-1-phosphate level. None of the patients showed obvious clinical symptoms suggesting that all had peripheral type galactosemia. The mean age of patients was 46 days (range: 14 days-11 months). Patients were subdivided into two groups according to their enzyme activities. Seven patients showed severely reduced enzyme activity of 2.82 ± 2.00 $\mu\text{mol/hour/gHb}$ (mean \pm SD, 0.8-6.2, Table 2), while the other 30 showed moderately reduced enzyme activity of 15.02 ± 4.55 $\mu\text{mol/hour/gHb}$ (mean \pm SD, 6.8-25.1) (mean \pm SD and normal range in the control subjects: 29.47 ± 7.71 , 19-47).

In order to investigate the underlying defects of GALE deficiency, the GALE gene was sequenced in the 7 Korean patients with severe GALE activity attenuation. Nine mutations were identified: 8 missense mutations (p.A25V, p.R40C, p.D69E, p.E165K, p.R169W, p.R239W, p.G302D, and p.R335H) and a single nonsense mutation (p.W336X). Small deletions or insertions were not detected. With the exception of p.R335H, all mutations proved novel. Six patients were compound heterozygotes and one patient had only one mutation; no homozygote was found (Table 2). PCR-RFLP was performed to confirm the above mutations. When amplified with the appropriate primers (described in Materials and Methods), the mutations either generated or destroyed enzyme restriction sites, as shown in Table 1. In order to exclude common polymorphisms for the described mutations, at least 100 alleles from healthy adult individuals without any clinical symptoms of galactosemia were screened for these nine nucleotide substitutions. No mutation was detected in normal individuals, which

Table 1
Restriction enzymes used to confirm the presence of the nine GALE mutations

Codon Change	Nucleotide Substitution	Restriction Enzyme	Product size (bp)	
			Wild-type	Mutation
p.A25V	c.74C>T	<i>BfaI</i>	181, 20	201
p.R40C	c.118C>T	<i>BtgI</i>	106, 118, 78	196, 106
p.D69E	c.207C>A	<i>Sau96I</i>	146, 51, 131	146, 182
p.E165K	c.493G>A	<i>TaqI</i>	213, 89	302
p.R169W	c.505C>T	<i>HpaII</i>	226, 76	302
p.R239W	c.715C>T	<i>HpaII</i>	65, 78, 80	143, 80
p.G302D	c.905G>A	<i>HphI</i>	132, 133	265
p.R335H	c.1004G>A	<i>HaeII</i>	119, 198	317
p.W336X	c.1007G>A	<i>NheI</i>	317	117, 200

Numbering according to GenBank Accession # NT L41668 (cDNA) and # NP_000394

Table 2

Mutation analysis in patients with severely reduced GALE activity

Patients (age)	Mutations	Enzyme activity in RBC ($\mu\text{mol/hr/gHb}$) ^a
1 (27 days)	p.G302D / p.R335H	0.8
2 (45 days)	p. R169W / p.A25V	1.5
3 (30 days)	p.D69E / p.G302D	1.7
4 (31 days)	p.D69E / p.E165K	1.7
5 (23 days)	p.R40C / p.R169W	2.9
6 (20 days)	p.W336X / not detected	4.9
7 (24 days)	p.R239W / p.R335H	6.2

^a Mean \pm SD in 7 patients, $2.82 \pm 2.00 \mu\text{mol/hr/gHb}$; Mean \pm SD and normal range in the control subjects, 29.47 ± 7.71 , $19\text{--}47 \mu\text{mol/hr/gHb}$

suggests that the reported mutations might be the cause of GALE deficiency.

In order to determine if the above mutations were present in the 30 moderately affected patients, we performed PCR-RFLP. Seventeen of these 30 patients were found to have one of the above mutations, and the remaining 13 did not. The detected mutations were p.G302D (7/17), p.R239W (5/17), p.R169W (3/17), p.D69E (1/17), and p.E165K (1/17). No significant difference was found between the 17 patients with mutations and the 13 without, in terms of average enzyme activity in RBCs (14.8 ± 5.3 vs. $15.4 \pm 3.6 \mu\text{mol/hour/gHb}$, mean \pm SD).

We also investigated the frequency of each mutation in 24 patients who had one or two mutant allele(s). Of the 30 mutant alleles detected in Korean patients with GALE deficiency galactosemia, three amino acid substitutions - p.G302D (9/30), p.R239W (6/30), and p.R169W (5/30) - were found to be most frequent and these accounted for 67% of all mutations. The other 33% comprised as follows: p.D69E (3/30), p.E165K (2/30), p.R335H (2/30), p.A25V (1/30), p.R40C (1/30), and p.W336X (1/30).

Interestingly, two of the most frequently mutated amino acids, p.G302 and p.R239, are located in a region that was previously shown to undergo major conformational change upon substrate binding by x-ray crystallography.¹⁵ Moreover, p.G302 and p.R239 are highly conserved among species, thus implying their functional importance.

DISCUSSION

Sequencing analysis of the coding region of GALE gene in 7 Korean patients with severe GALE activity attenuation identified nine mutations, of which eight were novel. None of these mutations were detected among at least 100 normal alleles, thus excluding the possibility that they represent common polymorphisms. Seventeen of the 30 patients with moderate GALE defects were also found to contain one of the above mutations, whereas the remaining 13 did not. However, since only the presence or absence of the above 9 mutations was tested, we cannot exclude the possibility that these 13 patients might have harbored other GALE mutations. The seven se-

verely affected patients selected for GALE gene sequencing analysis showed very low GALE activity in red blood cells, with an average of $2.8 \mu\text{mol/hour/gHb}$ (normal range, $19\text{--}47 \mu\text{mol/hour/gHb}$). Sequence analysis showed that 6 of the 7 were compound heterozygotes and that the seventh patient had only one mutation, namely, an amino acid substitution of tryptophan to a stop codon in exon 11 (p.W336X). The patient with a nonsense mutation also showed very low GALE enzyme activity ($4.9 \mu\text{mol/hour/gHb}$), which suggested that the second allele might also have been defective. It is possible that a large deletion or mutations in the promoter region existed that was not detected by our sequencing analysis. Alternatively, the truncated form of GALE protein may have acted in a dominant negative manner. Seventeen patients with mild GALE activity defects had heterozygous mutations in GALE gene i.e., p.G302D, p.R239W, p.R169W, p.D69E, and p.E165K. Since GALE activities in these patients were 50% ($14.8 \pm 5.3 \mu\text{mol/hour/gHb}$) of normal, it is likely that these mutants were non-functional and did not act as dominant negatives.

The structure of human epimerase, as determined by x-ray diffraction analyses suggests that the polypeptide folds into two distinct structural motifs: an N-terminal or nucleotide binding motif, and a C-terminal region responsible for binding the UDP-sugar substrate.¹⁶ The active site is positioned within the crevice between these two domains. The major conformational changes are limited to the regions defined by p.199Glu to p.240Asp and p.274Gly to p.308Tyr.¹⁵ Interestingly, the two most common mutations found in our Korean patients, p.R239W and p.G302D, are located in this region. Moreover, these two residues are highly conserved among species, suggesting the importance of these amino acids in structure and/or function of the enzyme. Other than p.R239W and p.G302D, none of the other mutations identified in the present study seems to be involved in substrate binding or to play a decisive role with respect to the active site. However, it is possible that changes in hydrophobicity (p.R169W), electrical charge (p.R40C, p.E165K and p.R169W), or size (p.A25V and p.D69E, and p.R335H) might cause conformational changes that affect enzyme activity. Structural analysis of the mutant proteins is required to explore these possibilities.

The functional consequences of the GALE proteins with selected mutations have been reported in terms of their enzyme activities, protein stabilities, and the phenotypic complementations using a yeast expression studies.^{7,11,17} When expressed in a yeast null background, p.N34S, p.G90E, p.V94M, and p.L183P severely affected GALE activity suggesting the importance of these highly conserved amino acid residues. However, the recent functional studies of p.K257R and p.G319E revealed that both substitutions may be polymorphism that exist in linkage disequilibrium with other, as yet unidentified causal mutations.¹⁷ Therefore, in order to determine the functional consequences of the newly identified substitutions in the present study, biochemical studies are mandatory and now under progress.

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