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Mutational Analysis of the *GALT* Gene in Filipino Patients

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Classic galactosemia is an inherited metabolic disorder due to mutations in the galactose-1-phosphate uridyltransferase (GALT) gene. This study describes the results of the GALT gene analysis of four unrelated Filipino patients with Classic Galactosemia. DNA extracted from dried blood spots and peripheral blood of the patients, age one month to two and a half years, underwent PCR-amplification with subsequent bidirectional sequencing of all eleven exons with their flanking intronic regions following standard protocols. Clinical data of these patients were reviewed. The patients presented with jaundice, hepatomegaly, diarrhea, vomiting, poor feeding and seizures during their neonatal period. They were diagnosed with elevated blood galactose and galactose-1-phosphate and absent GALT activity. Four missense mutations were found wherein two were previously reported (p.V168L and p.A345D) and two were novel (p.L116P and p.M178R). The most frequent mutation in our cohort is p.V168L. This study suggests that GALT mutations are ethnic-specific and that galactosemia is a heterogeneous disorder at the molecular level. The importance of early detection, immediate and proper medical management and genetic counseling of the patients and their families cannot be overemphasized.

INTRODUCTION

Galactosemia is an autosomal recessive disorder characterized by elevated blood levels of galactose (gal) and its metabolites due to enzyme deficiencies involved in its metabolism. Reactions in the pathway of gal metabolism are facilitated by three enzymes: galactokinase (GALK), gal-1-phosphate uridyltransferase (GALT) and UDP-gal 4-epimerase (GALE). The type and severity of the disease depend on the enzyme that is impaired.[1]

Lack of or severe reduction in GALT enzyme activity (EC 2.7.7.12) leads to classic galactosemia (OMIM# 230400), the most common and most severe form of the disorder that can be fatal if undiagnosed and not properly managed. Clinical manifestations include poor feeding, failure to thrive, vomiting, diarrhea, jaundice, sepsis and eventually, cataracts and mental retardation.[1–3] Dietary restriction of galactose results in clinical improvement. The birth incidence of classic galactosemia is 1:40,000-60,000 in Caucasian populations,[4] and about 1:600,000 in the Japanese.[5] In the Philippines, the combined prevalence of classic and non-classic galactosemia is 1:91,380 based on newborn screening data.[6]

Patients with classic galactosemia have elevated blood gal and gal-1-phosphate and urinary galactitol and galactonate. Sequence alterations in the *GALT* gene resulting to little or no enzyme activity are considered classic mutations (G), while the Duarte variant (D) reduces enzyme activity by approximately 25%.[7] There are numerous classic mutations that occur throughout the *GALT* gene, the most common of which are p.Q188R, p.S135L, p.K285N, p.L195P, p.Y209C, p.F171S, 5kbdel, and c.253-2A>G.[2] The Duarte mutation p.N314D in *cis* configuration with c.-119_-116delGTCA in the promoter region causes the impairment of a positive regulatory domain which in turn partially reduces the activity of the enzyme.[2] Therefore, biochemical data from compound heterozygous (D/G) individuals may give false positive newborn screening results. Molecular genetic testing has been useful in confirming the diagnosis of galactosemia and provides information that can lead to better management of the disease[8].

Galactosemia mutations are observed to be ethnically diverse. Asians have reportedly distinct mutations from Caucasians and African Americans.9This is the first study that aimed to determine the molecular basis of Classic Galactosemia by identifying the GALT mutations present among Filipinos diagnosed with the condition.

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MATERIALS AND METHODS

Subjects

Of five patients with classic galactosemia listed in the database of the Institute of Human Genetics-Metabolic Unit, covering the period 1996 to 2011, four patients were included in the study. They were either referred to the unit or detected by newborn screening and were confirmed by metabolic evaluation. GALT activity was quantified by Beutler test performed on a blood sample collected on to a Whatmann newborn screening filter card. The absence of GALT activity and elevated blood gal (normal: less than1.5 mmol/L in newborns; less than 0.5 mmol/L for older than newborn) established the diagnosis of classic galactosemia. The patient case records were reviewed. Signed informed consent was obtained from the patients' parents or legal guardians.

Blood Collection and DNA Extraction

The Ethics Review Committee of the National Institutes of Health Philippines approved all DNA testing. Collection of blood specimens was done during regular follow-up schedules of patients in the Pediatric Metabolic Genetics clinic, together with the routine blood extraction for clinical monitoring. Genomic DNA for PCR amplification was extracted either from punched discs (3mm)/dried blood spots in filter cards or from four mL whole blood in EDTA tubes using the Qiagen QIAamp DNA Blood Mini/Midi Kit (Qiagen, Santa Clara, CA). DNA extraction was carried out based on the manufacturer's protocol.

PCR Amplification and Agarose Gel Electrophoresis

DNA primers for the amplification of nine of 11 exons of the *GALT* gene were based on literature.[10] Primer pairs for exons one and 11 were designed using the Primo Pro 3.4 PCR Primer Design.[11]Polymerase chain reaction (PCR) was performed to amplify the exons and their flanking regions in 50-µL reaction volumes containing 20 ng gDNA, 1.25X PCR buffer, 3 mM $MgCl_2$, 0.25 mM dNTP mix (Clontech Laboratories, Palo Alto, CA), 2.5 U Taq DNA Polymerase (Invitrogen, Carlsbad, CA) and 0.5 µM forward and reverse primers using the iCycler thermal cycler (Bio-Rad Laboratories). PCR conditions were as follows: initial denaturation at 94^{o}C for seven minutes; 35 cycles of 94^{o}C for 30 seconds, 59^{o}C for 30 seconds and 72^{o}C for 30 seconds; and final extension at 72^{o}C for 10 minutes. Amplicon quality was checked by agarose gel electrophoresis. Two uL PCR product was loaded on to a 2% agarose gel with GelRed^{TM} nucleic acid stain (Biotium, Hayward, CA) and run at 100V for 35 minutes in a 0.5X TBE Buffer (Invitrogen, Carlsbad, CA) via Mupid-2 Mini Gel Migration Trough (Cosmo Bio Co. Ltd.).

Sequence Analysis

Bidirectional sequencing of PCR products was done using the Applied Biosystems 3730xl DNA analyzer. Mutations were identified by comparing the patient sample sequences with the published wild type *GALT* genomic DNA sequence (GenBank NG_009029.1) using the Sequencher 4.7 software (Gene Codes Corp., Ann Arbor, MI). Changes in nucleotide sequence were confirmed in their parents' DNA.

RESULTS

From 1996-2011, five Filipino patients were diagnosed with Classic Galactosemia. Four of these patients were included in the study. Of the four, one patient died at one month of age due to sepsis. At the time of diagnosis, jaundice (4/4), hepatomegaly (3/4), diarrhea (3/4), vomiting (1/4) and failure to thrive (2/4) were the manifestations. All four patients had gram negative sepsis. One patient had a family history of consanguinity. Only Patient 3 was diagnosed as GALT deficiency by newborn screening (NBS) and was treated immediately after diagnosis. At present, the three surviving study patients are on a gal-restricted diet, have normal gal metabolites and normal psychomotor development (Table I).

Table I. Clinical data and genotypes of the four patients with classic galactosemia

Patient (Sex)	Gestational age (Birth weight)	Consan-guinity	Onset age	Laboratory findings for diagnosis	Clinical course	Genotype
Patient 1 (Male)	38 weeks (3232 grams)	+	3 days old	Blood gal: > 8 mmol/L GALT activity: Not detectable	Jaundice and sepsis occurred in the first two weeks. Hepatomegaly was noted at 1 month. Treatment with gal-free diet started after diagnosis. At 2 years and 6 months, development was normal. Neither liver abnormality nor cataracts were observed.	p.V168L/ p.M178R
Patient 2 (Male)	37 weeks (2600 grams)	-	5 days old	Blood gal: 7 mmol/L GALT activity: not detectable	At 5 days, vomiting, diarrhea and jaundice occurred. Hepatomegaly, failure to thrive and sepsis were noted at 1 month. Treatment with gal-free diet started after diagnosis. At 2 years and 6 months, development and liver function were normal.	p.A345D/ -
Patient 3 (Male)	36 weeks (3000 grams)	-	4 days old	Blood gal: > 8 mmol/L GALT activity: not detectable	At 4 days, jaundice, diarrhea, seizures, irritability and poor suck occurred. Hepatomegaly and sepsis were noted at 7 days. Treatment with gal-free diet started after diagnosis. At 11 months, liver function was normal.	p.L116P/ p.L116P
Patient 4 (Female)	39 weeks (3700 grams)	-	5 days old	Blood gal: > 8 mmol/L GALT activity: not detectable	At 5 days, jaundice, vomiting, and diarrhea occurred. The patient died at 1 month due to sepsis.	p.V168L/ p.V168L

Two novel missense mutations (p.L116P and p.M178R) were identified in addition to two previously reported mutations (p.V168L and p.A345D). The location of these mutations and their number of occurrence are listed in Table II. The most observed mutation, a G-to-T transversion at c.502 that produces a codon change converting valine to leucine at amino acid 168 (p.V168L), was detected in three out of eight mutant alleles. Known Duarte mutations within the sequenced region (c.-119_-116delGTCA and p.N314D) were not found.

Table II. *GALT* mutations in Filipino Patients with Galactosemia

Mutation	Nucleotide Change	Position	Number of Alleles	References
p.L116P	c.347T>C	Exon 4	2	(Novel)
p.V168L	c.502G>T	Exon 5	3	14*
p.M178R	c.533T>G	Exon 6	1	(Novel)
p.A345D	c.1034C>A	Exon 10	1	16
Total			7	

*ARUP Scientific Resource for Research and Education: GALT database. Available at http://www.arup.utah.edu/database/GALT/GALT_welcome.php. Accessed 10 June 2013.

Figure 1 shows the sequence variations detected in this study. All occurred within the coding region of the *GALT* gene. Two patients had homozygous genotypes – p.L116P/p.L116P in Patient 3 (Figure 1A) and p.V168L/p.V168L in Patient 4 (Figure 1B). Mutation analyses in both parents of these patients showed heterozygous status at the affected loci and confirmed the homozygous findings in Patients 3 and 4. Patient 1 was heterozygous for p.V168L and p.M178R (Figure 1C and 1D). Only one G allele, p.A345D, was found in Patient 2 (Figure 1E). The pathogenic mutation in the other allele may be located outside the exonic region and exon-intron boundaries of the *GALT* gene.

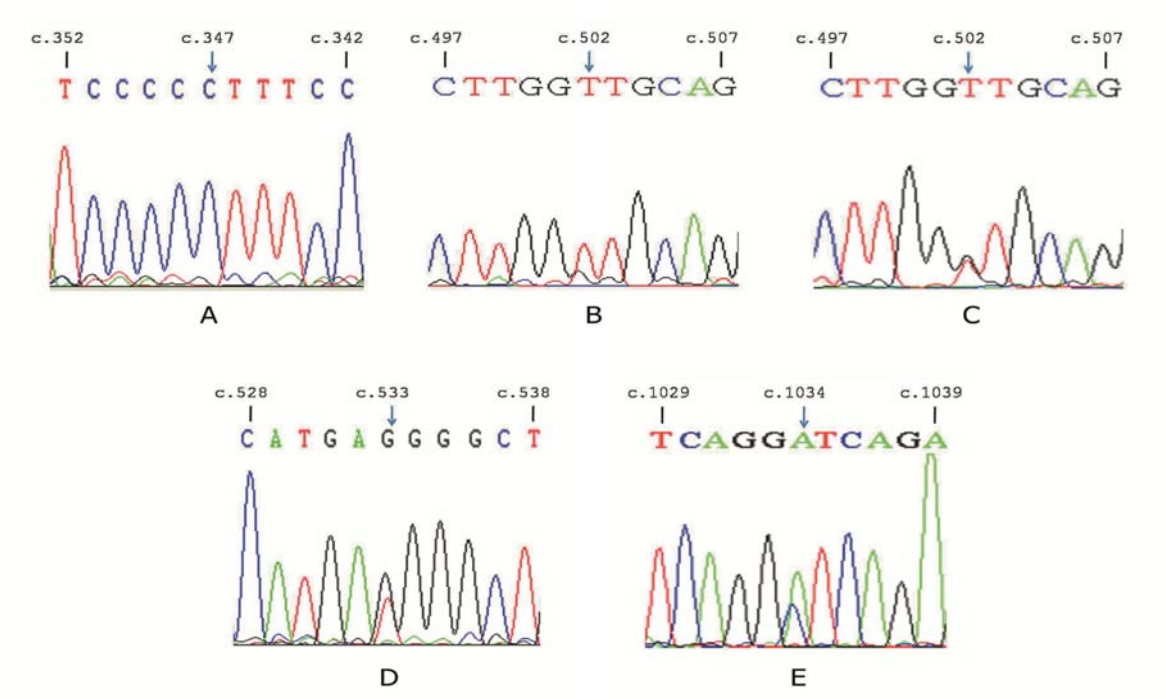

Figure 1. Electropherograms showing the *GALT* mutations identified in this study.
(A) homozygous T-to-C transition at c.347 (p.L116P) in exon 4
(B) homozygous G-to-T transversion at c.502 (p.V168L) in exon 5
(C) heterozygous G-to-T transversion at c.502 (p.V168L) in exon 5
(D) heterozygous T-to-G transversion at c.533 (p.M178R) in exon 6
(E) heterozygous C-to-A transversion at c.1034 (p.A345D) in exon 10

DISCUSSION

Classic galactosemia is an autosomal recessive disorder caused by a deficiency of GALT, the enzyme that catalyzes the metabolism of galactose-1-phosphate to uridine diphosphate galactose. The resulting elevation of gal-1-phosphate leads to intellectual retardation, liver dysfunction and cataract formation.[1–3] The diagnosis is usually made during the neonatal period. In the present study, the age at onset of symptoms is three to five days

and age at the time of diagnosis ranged from seven days to one month and 10 days indicative of significant delays in diagnosis. Noteworthy is that only one patient had the benefit of a newborn screen. The three other patients did not comply with newborn screening for unknown reasons. Awareness of the importance of early diagnosis through newborn screening cannot be overstated. It is also important that a high level of suspicion for galactosemia be present when a child with jaundice, hepatomegaly, diarrhea, poor feeding and seizures is encountered.

GALT, the second enzyme of the Leloir pathway, is coded by the *GALT* gene located in chromosome 9p13. The gene has a total length of approximately four kb and contains 11 exons.[2] Mutations at the *GALT* locus can either lead to a severe classic type or to a Duarte galactosemia, a mild to asymptomatic condition which is a more common clinical variant.[12] Classic mutations (G) cause severe or complete reduction of GALT activity.[7] A combination of two G alleles causes classic galactosemia while a mixed heterozygote of G allele and a Duarte variant (D) allele, D/G, which results in about 75% enzyme activity reduction, is associated with the Duarte variant galactosemia.[8,12]

Classic galactosemia exhibits substantial allelic heterogeneity and over 250 *GALT* gene mutations are recorded in different populations and ethnic groups worldwide.[13,14] The results of this study support previous conclusions that *GALT* mutations are ethnic-specific.[15] The p.Q188R (c.563A>G) mutation, which is most prevalent in European populations or in those of predominantly European descent and accounts for about 60-70% of mutant chromosomes,[13] is absent in our patients. This mutation is also uncommon amongst Japanese patients.[5] Other commonly cited mutations such as the p.K285N (c.855G>T) that is present in approximately 26% of mutant alleles in Eastern Europeans, p.S135L (c.404 C>T) which is prevalent in African Americans[7], and the IVS2-2 A>G splicing mutation identified exclusively in Hispanics[16] were not detected in our patients.

In this study, we identified two novel missense mutations (p.L116P in Patient 3 and p.M178R in Patient 1) and two previously reported missense mutations (p.V168L in Patient 4 and p.A345D in Patient 2). The p.A345D mutation found in Patient 2 was previously detected by Yang and colleagues in 2002. It had 1% allele frequency in Hispanic or White newborns with classic galactosemia.[16] The p.A345D allele might have arisen in the Filipino population due to its Spanish bloodline as a result of the three-century colonization of the Philippines by Spain.

The most frequent G mutation (p.V168L) in this study, occurring in 37.5% of mutant alleles (Patients 1 and 4), was reported by Calderon *et al.* to be found in a Filipino female patient presenting with bilirubinemia. She was homozygous for this mutation and had totally impaired GALT enzyme activity.[14] Patient 4 was also homozygous for this mutation and she succumbed to severe infection at 1 month of age.

Although we identified four mutations in our cohort, we did not see any clinical correlation probably because of the very limited sample size. The patients with homozygous mutations were just as sick at the onset as those who were heterozygous. All patients presented with jaundice and severe infection early (between days three and five), regardless of the mutation. Patient 3 was detected through newborn screening and therefore, was treated by day seven of life. The other three were treated at one month or later. The delay in treatment will potentially cause more negative consequences.

CONCLUSION

This study reveals that the *GALT* mutations that occur in affected Filipinos are different from the common mutations of the European population. Further, our limited data set suggests that p.V168L may be common among Filipino galactosemia patients. It emphasizes the heterogeneity of galactosemia at the molecular level and because of this, there is a potential use of mutational analysis of the *GALT* gene as a confirmatory diagnostic test. It can determine familial mutations, and is thus useful in carrier testing for at-risk relatives.[7] This study also stresses the importance of early detection, immediate and proper medical management and genetic counseling of the patients and their families.

The authors recommend mutation analysis for newly diagnosed Filipinos with classic galactosemia in order to identify the most common mutations that may be used to establish targeted analysis for confirmatory testing.

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