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Newborn Screening for Fabry Disease in Taiwan Reveals a High Incidence of the Later-Onset Mutation c.936+919G>A (IVS4+919G>A)

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Abstract

Fabry disease (α-galactosidase A (α-Gal A, GLA) deficiency) is a panethnic inborn error of glycosphingolipid metabolism. Since optimal therapeutic outcomes depend on early intervention, a pilot program was designed to assess newborn screening for this disease in 171,977 consecutive Taiwanese newborns by measuring their dry blood spot (DBS) α-Gal A activities and β-galactosidase/α-Gal A ratios. Of the 90,288 male screenees, 638 (0.7%) had DBS α-Gal A activity <30% of normal mean and/or activity ratios >10. A second DBS assay reduced these to 91 (0.1%). Of these, 11 (including twins) had <5% (Group-A), 64 had 5–30% (Group-B), and 11 had >30% (Group-C) of mean normal leukocyte α-Gal A activity. All 11 Group-A, 61 Group-B, and 1 Group-C males had *GLA* gene mutations. Surprisingly, 86% had the later-onset cryptic splice mutation c.936+919G>A (also called IVS4+919G>A). In contrast, screening 81,689 females detected two heterozygotes. The novel mutations were expressed *in vitro*, predicting their classical or later-onset phenotypes. Newborn screening identified a surprisingly high frequency of Taiwanese males with Fabry disease (~1 in 1,250), 86% having the IVS4+919G>A mutation previously found in later-onset cardiac phenotype patients. Further studies of the IVS4 later-onset phenotype will determine its natural history and optimal timing for therapeutic intervention.

Keywords

Fabry disease; α-galactosidase A deficiency; newborn screening; *GLA*

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Introduction

Fabry disease (MIM# 301500) is an X-linked lysosomal storage disorder resulting from the deficient activity of the lysosomal glycohydrolase α-galactosidase A (α-Gal A) [Desnick et al., 2001]. The enzymatic defect leads to progressive accumulation of globotriaosylceramide (GL-3) and related glycosphingolipids primarily in the vascular endothelium of the skin, kidney, heart, and brain. Affected males who have little or no α-Gal A activity exhibit the classic phenotype with onset of angiokeratomas, acroparesthesias, and hypohidrosis in childhood [Desnick et al., 2004]. With advancing age, the occurrence of renal failure, cardiac disease and stroke lead to premature death. The clinical manifestations in heterozygous women with mutations causing the classic phenotype range from asymptomatic to as severe as those in affected males. To date, over 500 disease-causing mutations in the α-Gal A gene (*GLA*; MIM# 300644) have been identified [Stenson et al., 2003]. The mutations include missense, nonsense, various frame-shift lesions (small deletions, insertions, and rearrangements), and splicing mutations [Desnick et al., 2001]. Most mutations are "private," occurring in one or a few affected families.

The incidence of classic Fabry disease was initially estimated to be 1 in 40,000 to 60,000 males [Meikle et al., 1999; Desnick et al., 2001]. However, during the past decade the clinical spectrum of Fabry disease expanded from the classic phenotype to include the later-onset phenotype detected in males with renal, cardiac, and cerebrovascular disease [von Scheidt et al., 1991; Desnick et al., 2001; Nakao et al., 1995; Nakao et al., 2003; Rolfs et al., 2005]. Screening by plasma α-Gal A activity identified previously undiagnosed Fabry patients in 0.25% to 1% of males undergoing hemodialysis [Nakao et al., 2003; Kotanko et al., 2004; Tanaka et al., 2005;], 3% to 4% of males with left ventricular hypertrophy or hypertrophic cardiomyopathy [Nakao et al., 1995; Sachdev et al., 2002; Monserrat et al., 2007], and up to 4.9% of males with acute cryptogenic strokes [Rolfs et al., 2005]. In a recent study, newborn screening of over 37,000 consecutive males in Italy identified 12 infants with α-Gal A mutations including 11 who had lesions that expressed residual activity consistent with the later-onset phenotype [Spada et al., 2006]. In the Italian study, the frequencies of newborns predicted to have the classic and later-onset phenotypes were ~1 in 37,000 and 1 in 3,373 males, respectively, while the overall frequency of Fabry disease was ~1 in 3,100 Caucasian males.

Recently, recombinant α-Gal A replacement therapy has been shown to clear microvascular endothelial deposits of GL-3 from the kidneys, heart, and skin in patients with Fabry disease, reversing the primary pathogenesis of the clinical manifestations of this disease [Eng et al., 2001; Wilcox et al., 2004; Banikazemi et al., 2007; Germain, 2007]. These studies revealed that early treatment, before significant renal impairment (glomerular filtration rate <60 ml/min) [Wilcox et al., 2004; Banikazemi et al., 2007; Germain, 2007] or cardiac disease [evidence of late enhancement on magnetic resonance imaging (MRI)] [Moon et al., 2003; Weidemann et al., 2003], is important for optimal therapeutic outcomes. Although early treatment is critical in Fabry disease to preserve organ function, many patients are diagnosed late after significant disease progression, due to the non-specific nature of the clinical manifestations early in the disease [Desnick et al., 2003; Eng et al., 2006]. Common Fabry symptoms in childhood, such as gastrointestinal problems, acroparesthesias, and hypohidrosis, can easily be attributed to other etiologies or even be regarded as psychological problems [Desnick et al., 2003]. Additionally, the early symptoms of the classic phenotype are usually absent in patients with the later-onset phenotype [von Scheidt et al., 1991; Desnick et al., 2001; Nakao et al., 1995; Nakao et al., 2003; Rolfs et al., 2005]. Even when diagnosed later in life, enzyme replacement therapy has been shown to slow the disease progression [Banikazemi et al., 2007].

To determinate the frequency of the disease in an Asian population, newborn screening was undertaken in over 170,000 consecutive male and female Taiwanese babies. We identified newborns carrying both reported and novel α-Gal A gene mutations. By evaluation of at-risk consenting family members, previously undiagnosed individuals affected by Fabry disease were identified. Of note, a high frequency (~1 in 1,410) of the g.9331G>A (c.936+919G>A; often called IVS4+919G>A) cryptic splice mutation, previously reported in Japanese cardiac variants [Ishii et al., 2002], was identified in the Taiwanese newborn males.

Materials and methods

Newborn screening

For 24 months (July, 2006 through June, 2008), 171,977 consecutive male and female newborns were screened for α-Gal A deficiency on day 3 of life. This pilot program was conducted by the Newborn Screening Center at the National Taiwan University Hospital (NTUH), which screens approximately 40% of all newborns in Taiwan. The dried blood spots (DBS) used in this study were punched from routine newborn filter cards. This study was approved by the Institutional Review Board of the National Taiwan University Hospital, and informed consent was obtained from the parents of participating newborns.

Screening Assay on DBS

To determine α-Gal A activities in the DBS, assays were carried out essentially as previously described for total α-Gal A activity [Chamoles et al., 2001], and for β-galactosidase (β-Gal) activity [Lukacs et al., 2005] which served as a control enzyme. A two-tiered format was employed (Figure 1): samples which had α-Gal A activities <55% of the normal mean (5.87 μmol/L whole blood/h, SD=3.04, range 0.6 – 50.97 μmol/L/h, n=6947) were analyzed again for both α-Gal A and β-Gal activities and an β-Gal/α-Gal A activity ratio was determined (normal mean ratio = 5.01 ± 1.56, range 1.21–11.30, n=606). For samples with α-Gal A activities <30% and/or a ratio >10, a second newborn filter card was requested. If the second DBS had an α-Gal A activity <30% and/or a ratio >10 in males, or an α-Gal A activity <30% and a ratio >20 in females, the respective newborn's parents would be asked for a blood sample from the infant to determine the leukocyte α-Gal A activity. The cut-offs for females were stricter since it is more difficult to diagnose Fabry disease by enzyme assay in women due to random X-inactivation [Dobrovolny et al., 2005; Maier et al., 2006].

Confirmatory α-Gal A Assay in Isolated Leukocytes

Leukocyte α-Gal A activities were determined with the fluorogenic substrate, 4-methylumbelliferyl-α-*D*-galactopyranoside (Sigma-Aldrich, MO, USA) in the presence of α-*N*-acetyl-*D*-galactosamine (Sigma-Aldrich, MO, USA) to inhibit α-*N*-acetylgalactosaminidase activity [Mayes et al., 1981]. The diagnosis of Fabry disease was based on a leukocyte α-Gal A activity ≤ 4.76 nmol/hr/mg protein (5% of normal mean; normal mean ± SD = 95.1 ± 30.3 nmol/hr/mg protein; n=40).

For newborns with low leukocyte α-Gal A activities, family pedigrees were obtained, and at-risk family members were contacted, counseled and asked to provide blood samples for diagnostic studies, with informed consent. Genomic DNAs were isolated from whole blood, and each of the seven exons and their flanking intronic sequences of *GLA* gene (NC_000023.9; GI:89161218) were amplified as previously described [Shabbeer et al., 2006] (protocol available on request). Each amplicon was then sequenced with an ABI Prism 3700 Capillary Array Sequencer, using the ABI Prism BigDye Terminator Ready Reaction Mix (Perkin-Elmer-Cetus). Mutations were confirmed by repeat amplification and sequencing from the opposite strand and by cosegregation of the lesion and disease in other

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family members. The cDNA (NM_000169.1) was numbered with +1 corresponding to the A of the ATG translation initiation codon (www.hgvs.org/mutnomen). The initiation codon is codon 1. The frequency of the IVS4 cryptic splicing mutation in the Taiwanese population was estimated by amplification of intron 4 and *Bfa*I cleavage of genomic DNAs isolated from 428 normal X-chromosomes from unrelated individuals (130 males and 149 females) [Ishii et al., 2002], but no IVS4+919G>A allele was detected. Urinary GL-3 was measured to determine the relative amount of storage material [Kitagawa et al., 2005].

Mutagenesis and in vitro expression

Site-specific mutagenesis of the wild-type α-Gal A cDNA was performed as previously described [Yasuda et al., 2003]. Each mutated cDNA was subcloned into pcDNA3.1/myc-his vector (Invitrogen, CA, USA), and the expression constructs were confirmed by sequencing. Eight *GLA* missense mutations and one in-frame three base deletion identified in the newborns, as well as the wild-type *GLA* cDNA, were expressed *in vitro*. Plasmid DNA (0.5 μg) was transfected into COS-1 cells grown in 6 cm dishes with Transfectamine-2000 (Invitrogen, CA, USA). Cells were harvested 40 hr after transfection and the α-Gal A activities were determined as described above for leukocytes.

To determine the stability of the expressed α-Gal A wild-type and mutant enzymes, the COS-1 cells were lysed and the expressed human α-Gal A activities in the cell lysates were incubated at pH 4.6 and 37°C and aliquots were removed at intervals over a 180 minute period for assay as described above. For each expressed enzyme activity, the stability profile was fitted for first order exponential decay kinetics [Desnick et al., 2001], and the half-lives were determined using the data analysis software of the OriginPro Program v.8 (Northampton, MA, USA).

Results

Newborn Screening

Normal DBS α-Gal A activity was determined in 6,947 consecutive newborns (52.5% males). The mean and median α-Gal A activities were 5.87 and 5.26 μmol/hr/L whole blood. DBS enzyme activities were normally distributed from 10.2% to 560% of the mean (Figure 2). The mean values of DBS α-Gal A activities for normal males and females were not statistically different.

Among the 171,977 consecutive male and female newborns screened, 963 (0.63%), including 325 females, had <30% of the mean normal DBS α-Gal A activity and/or a β-Gal/α-Gal A ratio >10, and were recalled for a second DBS sample. Of these, 94, including three females, (0.055% of the total population) were recalled to determinate their leukocyte α-Gal A activities (Figure 1). As indicated in Table 1, 11 males of the 89 newborns who provided samples had leukocyte α-Gal A activities <5% of the mean normal activity (Group-A), 64 males and two females had activities between 5 and 30% (Group-B), and 11 males and one female had activities >30% of mean normal activities (Group-C).

Mutation analysis and in vitro expression

Mutation analysis was performed to identify which newborns with decreased α-Gal A activities had gene mutations. All 11 Group-A newborns, who had <5% of normal mean leukocyte α-Gal A activity, were males who had α-Gal A mutations (Table 1). Proband 1 had the previously reported c.277G>A (p.D93N) mutation found in patients with the classical phenotype [Ishii et al., 2002;Lukacs et al., 2005]. Probands 2, 3, and 4 had the cryptic splicing mutation IVS4+919G>A previously described in Japanese patients with the later-onset cardiac phenotype [Ishii et al., 2002]. The other seven male newborns had novel

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mutations including an in-frame 9 bp deletion [c.34_42del (p.C12_L14del)] in the enzyme's leader sequence (Proband 5), and novel missense mutations c.1078G>T (p.G360C) in Proband 6 and twin Probands 7 and 8, c.137A>C (p.H46P) in Proband 9, c.358C>G (p.L120V) in Proband 10, and c.656T>C (p.I219T) in Proband 11.

In the 66 Group-B newborns (64 males, 2 females) who had α-Gal A activities between 5 and 30% of mean normal leukocyte activity, 59 males and one female had the IVS4+919G>A mutation (Probands 12–71), two males (Probands 72 and 73) had the novel c.1067G>A (p.R356Q) mutation, and one female (Proband 74) was heterozygous for the c.1078G>T (p.G360C) lesion. No mutation was detected in three males who had α-Gal A leukocyte activities of 11.5 to 16.2% of normal mean leukocyte α-Gal A activity.

Among the 12 Group-C newborns (11 males, 1 female) with leukocyte α-Gal A activities higher than 30% of normal mean, and/or a β-Gal/α-Gal A ratio >10 for males and >20 for females, there was only one male (Proband 75) who had a leukocyte α-Gal A activity of 56.9%, but had a β-Gal/ α-Gal A ratio of 10.6. He was found to have a previously reported mutation, c.196G>C (p.E66Q), which was identified in patients with later-onset renal disease [Ishii et al., 1992].

All five novel missense mutations predicted amino acid substitutions (p.H46P, p.L120V, p.I219T, p.R356Q, and p.G360C) that occurred at highly conserved residues in the α-Gal A protein (Figure 3), and none was detected in 400 X-chromosomes from normal Taiwanese individuals. The c.34_42del (p.C12_L14del) mutant enzyme was an in-frame deletion of three amino acid residues that occurred in the leader sequence, but had markedly reduced α-Gal A activity. In vitro expression in COS-1 cells revealed that mutant enzymes p.C12_L14del and p.G360C had very low α-Gal A activities (4% and 6% of mean wild-type expressed activity) (Table 2) predicting that these mutations cause the classic phenotype. In contrast, mutant enzymes p.H46P, pE66Q, p.L120V, p.I219T, and p.R356Q had significant residual activities in the COS-1 cells, 36%, 52%, 42%, 46%, and 15% of expressed mean wild-type activity, respectively. Compared to the half-life (4.3 hr) of the expressed wild-type activity at 37°C and pH 4.6, mutant enzymes p.H46P, p.E66Q, p.L120V, p.I219T, and p.R356Q had half-lives of 1.5, 0.7, 1.9, 8.0, and 1.5 hr, respectively (Table 2).

In sum, 72 male (not counting an affected twin) and two female newborns had low α-Gal A enzymatic activities and α-Gal A mutations, an overall frequency of ~1 in 1,250 males and ~1 in 40,840 females. Four males (counting twin Probands 7 and 8 as 1), who had the predicted mutant proteins, p.D93N, p.G360C, and p.C12_L14del, that expressed very low α-Gal A activities, were predicted to have the classic phenotype, a frequency of about 1 in 22,570 newborn males. The six males with later-onset missense mutations and the 59 male newborns with the IVS4 cryptic splicing mutation gave an estimated frequency of the later-onset phenotype of ~1 in 1,390 male newborns. The IVS4 cryptic splicing mutation was detected in ~1 in 1,460 male newborns.

Family studies

Although privacy is highly regarded in Taiwanese culture, medical privacy is even more respected. When asked for information about family members, only three families would provide information for publication, two with classic mutations and one with a later-onset lesion. All three families had previously undiagnosed symptomatic family members. Proband 1, who had the c.227G>A (p.D93N) classic phenotype mutation, had a large number of maternal relatives, including five males who had histories of acroparesthesias and hypohidrosis, but had not been previously diagnosed as having Fabry disease. As shown in Figure 4 (upper panel), III-1, a 39 year old male, had a history of severe acroparesthesias since early childhood which lead to school problems due to frequent long absences for

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unexplained pain episodes. He had a stroke at age 35 and was hemiparetic for two years. Brain MRI revealed old infarctions. He had difficulty ambulating, had bilateral ventricular hypertrophy on echocardiography, and renal involvement with a decreased creatinine clearance (79 ml/min/1.73m^2) and proteinuria (1.3 g/day). A renal biopsy revealed the typical Fabry pathology. III-3, a 39 year old uncle, had a similar history of acroparesthesias, hypohidrosis and heat intolerance during childhood. Angiokeratoma were present in the typical distribution. He had microalbuminuria (microalbumin/creatinine = 0.05) and a renal biopsy revealed the typical pathology.

The grandmother of Proband 11, who had the c.656T>C (p.I219T) mutation, was a 54 year old heterozygote who had been on hemodialysis for end-stage renal disease for two years (Figure 4, lower panel). Proband 74, a newborn female with 11.9% of normal mean leukocyte α-Gal A activity, inherited the mutation from her 33 year old father who had markedly decreased (2.7% of normal mean) leukocyte α-Gal A activity, a normal EKG, and no proteinuria.

Biochemical identification of Classic and Later-Onset Phenotypes

The elevated β-Gal A/ α-Gal A ratios in newborn males with mutations predicting the classic phenotype ranged from 48.4 to 284, whereas the missense mutations and IVS4 splice mutation predicting the later-onset phenotype had ratios ranging from 10.6 to 135 and 6.14 to 76.4, respectively [data not shown]. Thus, the ratios overlapped between the two phenotypes. Urinary GL-3 levels were measured in five Group-A patients who provided specimens (Table 1, 3 classic and 2 later-onset mutations). Compared to the mean and range from 30 normal newborns (0.17 ± 0.13 μg/mg creatinine), all five newborns had elevated GL-3 levels. The three newborns predicted to have the classic phenotype had GL-3 levels of 11, 33.7 and 74 μg/mg creatinine, which could not be discriminated from those of the two infants predicted to have the later-onset phenotype who had levels of 11 and 19 μg/mg creatinine.

Discussion

Newborn screening provides the opportunity to determine the incidence of an inherited disease, to prospectively document the early disease manifestations, and to initiate therapy prior to irreversible organ damage. In 2006, Spada and co-workers reported the screening of 37,104 consecutive Italian newborn males for Fabry disease by determining their DBS α-Gal A activities and found that 12 neonates, or 1 in 3,100 males, had deficient α-Gal A enzyme activities and confirmed α-Gal A gene mutations [Spada et al., 2006]. Mutation analysis predicted that one of the newborns had the classic phenotype (1 in ~37,000), whereas 11 of the newborns were predicted to have the later-onset phenotype (1 in ~3,400).

Screening of 1 7 1,977 consecutive Taiwanese newborns for Fabry disease by determination of their DBS α-Gal A activities detected a total of 75 newborns, including two females, who had low leukocyte α-Gal A activities and α-Gal A mutations (Table 1). Of the 90,288 male newborns screened, 72 (considering the affected twins as 1) had α-Gal A mutations for a frequency of ~1 in 1,250, about 2.5 times more frequent than that found in the Italian newborns [Spada et al., 2006]. Six novel α-Gal A mutations were detected among these newborns. The five novel missense mutations and the in-frame deletion of three amino acids in the leader sequence occurred in conserved amino acid residues (Figure 3) and were absent in over 400 normal control alleles. In vitro expression and enzyme stability studies (Table 2) revealed that the previously reported mutant enzyme p.D93N and novel mutant enzymes p.G360C and p.C12_L14del had very low α-Gal A activities, predicting the classic phenotype. The other two previously reported mutations, c.196G>C (p.E66Q) and IVS4+919G>A, and four novel mutations, c.137A>C (p.H46P), c.358C>G (p.L120V), c.

656T>C (p.I219T) and c.1067G>A (p.R356Q), had residual activities and stabilities that predicted the later-onset phenotype.

Of particular note, was the surprisingly high prevalence (~1 in 1,460 males) of the IVS4+919G>A splicing mutation in the Taiwanese newborns. This mutation was first discovered in Japanese patients with the later-onset cardiac phenotype who had ~10% residual α-Gal A activity in lymphoblasts [Ishii et al., 2002]. The G to A transversion enhanced the percent expression of an alternatively spliced α-Gal A variant that was normally expressed at low levels, and included a 57-nucleotide intronic sequence that caused a frameshift mutation, resulting in a truncated enzyme polypeptide of 222 amino acid residues that had no detectable enzymatic activity. The amount of the wild-type transcript was markedly reduced to <10% of the wild-type expression. In the study by Ishii and colleagues, 1,603 consecutive male patients with non-specific cardiac symptoms were screened and the 230 who had left ventricular hypertrophy had their plasma α-Gal A activities determined. Seven (3%) had markedly decreased plasma α-Gal A activities and five of the seven had the IVS4+919G>A mutation [Ishii et al., 2002]. Since the entire intronic regions are not routinely evaluated when sequencing α-Gal A, the occurrence of the IVS4+919G>A and other intronic disease-causing lesions may be underestimated. The relatives in the newborn's families and other patients with the IVS4 mutation should be clinically followed in order to document the severity and variability of this later-onset genotype. A recent study suggested that the intronic lesion g.9273C>T also caused a similar splicing defect which resulted in clinical manifestations of Fabry disease [Filoni et al., 2008].

Thus, in Taiwan, the overall detection of mutation-confirmed males with Fabry disease was ~1 in 1,250 with frequencies of the classic and later-onset phenotypes of ~1 in 22,570 males and ~1 in 1,390 males. The disease was more frequent than in the Italian newborn study which detected an overall incidence of 1 in 3,080 males with predicted classic and later-onset frequencies of ~1 in 37,800 and ~1 in 3,400 males, respectively [Spada et al., 2006].

Notably, screening over 80,000 newborn females resulted in the detection of only two heterozygotes, one with a classic and the other with the IVS4 later-onset mutation. Based on the prevalence of the IVS4 mutation in newborn males, the sensitivity for detecting heterozygotes for this cryptic splicing lesion was unexpectedly low. These findings indicate that newborn screening for Fabry disease is markedly more effective and cost beneficial when screening is limited to male newborns. Although the natural history of the later-onset phenotype has not been systematically documented in female heterozygotes, most appear to have minimal life-threatening manifestations.

Recognizing the ethical and psychosocial issues raised by the identification of the later-onset variants among the newborn males, efforts were directed to predict the disease phenotype in the first DBS screening by determining the β-Gal/α-Gal A ratios and plotting them against the α-Gal A activity. In addition, the urinary GL-3 levels were determined in a few newborn males who had low enzyme activities (<5% of the normal leukocyte mean). Neither individually nor together did these determinations reliably predict newborns to have the classic or later-onset phenotype. To date, the most reliable predictor is the specific α-Gal A mutation. Clearly, expert laboratories and clinicians should establish a genotype/phenotype registry to document the clinical manifestations of each genotype. If modifier genes are identified, these too should be part of the genotype/phenotype predictions. In this way, newborn screening for Fabry disease could focus on males with the classic phenotype who would benefit from early therapeutic intervention.

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In summary, newborn screening for Fabry disease in Taiwan identified a high frequency of Fabry disease (~1 in 1,250 males) of which the majority (86%) had the later-onset phenotype (~1 in 1,390 males). These studies highlight the fact that newborn screening can identify large numbers of patients who will remain clinically asymptomatic until later in life. Although investigation of family members may identify older affected relatives with this X-linked disease who may benefit from therapeutic intervention, the newborn diagnosis of infants presents predicted to have the later-onset phenotype is controversial. This concern will become even more problematic for common later-onset disorders when multi-disease chip analyses or whole genome sequencing become commonplace for prospective parents and newborns. Clearly, well-documented genotype/phenotype registries will become essential for the future selection of patients for early therapeutic intervention.

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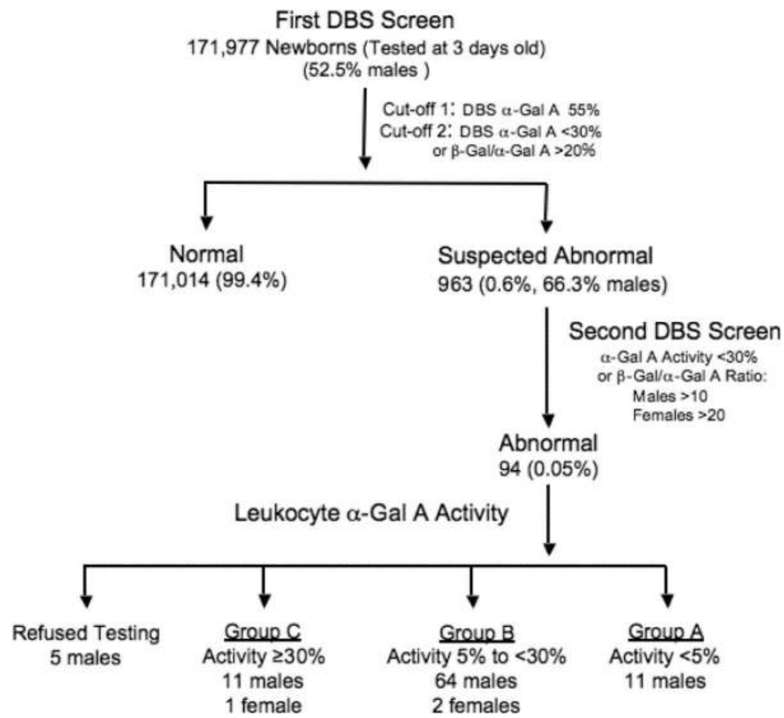

Figure 1.

Scheme used to identify and confirm α-Gal A deficiency in newborns. Note cut-offs for male (M) and female (F) newborns differ. The number and percentage of newborns detected under each category are indicated.

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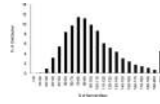

Figure 2.
Distribution of α-Gal A activity in 606 normal newborns. The X- and Y-axes indicate the percent (%) of normal mean and the percent (%) of the distribution for each activity percentage, respectively.

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	p.C12_L14del	p.H46P	p.L120V
Human	R N P E L H L G C A L A L	T M G W L H W E R F	P H G I R Q L A N Y
Rhesus	R N P E M H L G C A L A L	T M G W L H W E R F	P H G I R Q L A N Y
Mouse	L S R D T R L V C E L A L	T M G W L H W E R F	P S G I K H L A N Y
Dog	K S Q L L Q P C F L L A L	T M G W L H W E R F	P N G I Q S L A N Y
Horse	R S R A Q L L G C V L G L	T M G W L H W E R F	P G G I R R L A D Y
Armadillo	A S R G S W L N R A I S L	T M G W L H W E R F	P G G I H R L A N Y
Opossum	D H P G Q E R G P M F A F	T M G W L H W E R F	P S G I R H L A D Y
Platypus		P M G W L A W E R F	P G G I R R L A N Y
Lizard		P M G W L H W E R F	P S G I G K L A D Y
Chicken		P M G W L H W E R F	P S G I R A L A D Y
X. tropicalis			P G G I K K L A D Y
Stickleback		T M G W L H W E R F	P G G I K K L A D Y

	p.I219T	p.R356Q	p.G360C
Human	N Y T E I R Q Y C N	A M I N R Q E I G	N R Q E I G G P R S
Rhesus	N Y T E I R Q Y C N	A M I N R Q E I G	N R Q E I G G P R S
Mouse	N Y T D I Q Y Y C N	A V R N L Q E I G	N L Q E I G G P C P
Dog	N Y T E I R Q Y C N	A M V N L Q E I G	N L Q E I G G P R F
Horse	N Y T E V R E Y C N	A M V N L Q E I G	N L Q E I G G P R F
Armadillo	N • V D T C • Y - -	A I V N L R E I G	N L R E I G G P R S
Opossum	H Y T E I R Q Y C N	A V L N Q K E I G	N Q K E I G G P Q N
Platypus	N Y T E I R Q Y C N	A M V N R Q E I G	N R Q E I G G P Q G
Lizard	N Y T E I K Q Y C N	A V V N R Q E I G	N R Q E I G G P Q A
Chicken	N Y T E I K Q Y C N	A V L N Q Q E I G	N Q Q E I G G P Q N
X. tropicalis	N Y S E V A E Y C N	A V T N R N E I G	N R N E I G G P R N
Stickleback	N Y T A I R E A C N	A V I N M L E I G	N M L E I G G S R R

Figure 3.

Phylogenetic conservation of wild-type human α-Gal A amino acid residues, highlighting the amino acids substituted in mutant enzymes p.C12_L14del, p.H46P, p.L120V, p.I219T, p.R356Q, and p.G360C due to the novel missense mutations identified in the newborns.

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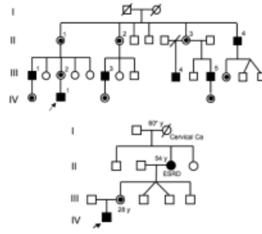

Figure 4.

Pedigrees of two Fabry families detected by newborn screening: a) Proband 1, affected males and obligate female heterozygotes are indicated, and b) Proband 11, note that the maternal grandmother had end stage renal disease (ESRD).

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Table 1

Baseline characteristics for α-Gal A mutation-confirmed newborns grouped by leukocyte α-Gal A activity[2]

Group[a]	Proband No	Sex	1st spot α-Gal Activity	1st spot β-Gal/ α-Gal A Ratio	Leukocyte α-Gal A Activity	GLA Mutation	Predicted effect	Urine GL-3 (μg/mg creatinine)
A[a]	1	M	5.13%	169.0	1.07%	c.277G>A	p.D93N	74.0
	2	M	11.5%	40.6	3.55%	g.9331G>A[b]	[c]	-
	3	M	27.1%	9.6	4.03%	g.9331G>A		-
	4	M	19.3%	19.2	4.67%	g.9331G>A		-
	5	M	1.87%	284.0	3.26%	c.34_42del	p.C12_L14del	33.7
	6	M	17.4%	48.4	3.05%	c.1078G>T	p.G360C	11.7
	7	M	5.75%	62.4	3.46%	c.1078G>T	p.G360C	-
	8	M	4.20%	61.4	3.63%	c.1078G>T	p.G360C	-
	9	M	12.3%	63.8	3.57%	c.137A>C	p.H46P	-
	10	M	7.15%	135.0	4.42%	c.358C>G	p.L120V	11.0
	11	M	45.6%	99.7	4.47%	c.656T>C	p.I219T	19.2
B[a]	12	M	16.0%	34.3	6.22%	g.9331G>A		-
	13	M	12.7%	10.9	7.15%	g.9331G>A		-
	14	M	9.02%	35.1	7.23%	g.9331G>A		-
	15	M	12.9%	17.0	7.44%	g.9331G>A		-
	16	M	23.3%	32.5	7.90%	g.9331G>A		1.59
	17	M	25.0%	20.3	8.45%	g.9331G>A		-
	18	M	19.3%	20.7	8.74%	g.9331G>A		-
	19	M	41.7%	28.9	8.75%	g.9331G>A		-
	20	M	10.9%	34.2	9.07%	g.9331G>A		2.62
	21	F	11.7%	35.9	9.20%	g.9331G>A heterozygote		-
	22	M	18.8%	24.9	9.40%	g.9331G>A		-
	23	M	22.9%	26.5	9.49%	g.9331G>A		0.55
	24	M	18.8%	46.9	9.63%	g.9331G>A		-
	25	M	23.2%	25.0	9.69%	g.9331G>A		3.33
	26	M	8.55%	22.6	9.71%	g.9331G>A		7.38

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Group[a]	Proband No	Sex	1st spot α-Gal Activity	1st spot β-Gal/ α-Gal A Ratio	Leukocyte α-Gal A Activity	GLA Mutation	Predicted effect	Urine GL-3 (μg/mg creatinine)
	27	M	13.5%	18.8	9.82%	g.9331G>A		-
	28	M	11.7%	19.8	10.3%	g.9331G>A		2.12
	29	M	16.8%	36.7	10.4%	g.9331G>A		3.85
	30	M	35.0%	35.7	10.5%	g.9331G>A		-
	31	M	6.38%	39.5	10.9%	g.9331G>A		1.32
	32	M	43.7%	34.9	11.3%	g.9331G>A		9.25
	33	M	45.7%	30.9	11.4%	g.9331G>A		-
	34	M	16.3%	23.4	11.5%	g.9331G>A		-
	35	M	27.7%	12.8	11.6%	g.9331G>A		1.77
	36	M	11.7%	14.0	11.7%	g.9331G>A		-
	37	M	16.9%	10.0	11.9%	g.9331G>A		-
	38	M	25.0%	27.0	12.1%	g.9331G>A		3.69
	39	M	37.9%	31.2	12.2%	g.9331G>A		1.45
	40	M	14.9%	37.2	12.3%	g.9331G>A		-
	41	M	37.3%	6.14	12.3%	g.9331G>A		-
	42	M	24.7%	39.6	13.0%	g.9331G>A		-
	43	M	21.5%	40.7	13.0%	g.9331G>A		3.79
	44	M	19.6%	42.6	13.0%	g.9331G>A		-
	45	M	49.0%	21.7	13.2%	g.9331G>A		3.17
	46	M	48.4%	39.3	13.4%	g.9331G>A		-
	47	M	34.4%	23.9	13.5%	g.9331G>A		-
	48	M	30.5%	27.2	13.7%	g.9331G>A		-
	49	M	16.3%	22.6	13.9%	g.9331G>A		-
	50	M	19.4%	25.8	14.1%	g.9331G>A		-
	51	M	19.1%	26.5	14.3%	g.9331G>A		-
	52	M	4.51%	76.4	14.6%	g.9331G>A		-
	53	M	21.8%	15.0	14.7%	g.9331G>A		-
	54	M	20.8%	42.5	14.8%	g.9331G>A		0.99
	55	M	15.9%	38.3	14.9%	g.9331G>A		-
	56	M	20.5%	27.2	15.8%	g.9331G>A		5.35

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Group[a]	Proband No	Sex	1st spot α-Gal Activity	1st spot β-Gal/ α-Gal A Ratio	Leukocyte α-Gal A Activity	GLA Mutation	Predicted effect	Urine GL-3 (μg/mg creatinine)
	57	M	35.3%	15.7	16.1%	g.9331G>A		5.42
	58	M	19.0%	32.5	17.7%	g.9331G>A		3.75
	59	M	23.2%	17.5	18.2%	g.9331G>A		-
	60	M	17.4%	37.8	18.6%	g.9331G>A		-
	61	M	34.5%	30.9	18.9%	g.9331G>A		-
	62	M	23.5%	13.9	19.1%	g.9331G>A		-
	63	M	41.2%	21.8	19.8%	g.9331G>A		4.56
	64	M	16.9%	38.0	20.4%	g.9331G>A		-
	65	M	14.0%	35.5	21.0%	g.9331G>A		-
	66	M	49.5%	29.7	21.8%	g.9331G>A		3.02
	67	M	33.3%	50.5	22.3%	g.9331G>A		3.1
	68	M	21.5%	41.5	22.3%	g.9331G>A		-
	69	M	23.6%	50.5	22.4%	g.9331G>A		-
	70	M	14.0%	21.2	24.5%	g.9331G>A		-
	71	M	22.7%	33.4	28.5%	g.9331G>A		0.92
	72	M	17.1%	51.5	12.5%	c.1067G>A	p.R356Q	-
	73	M	18.2%	43.8	19.1%	c.1067G>A	p.R356Q	-
	74	F	21.8%	20.7	11.9%	c.1078G>T heterozygote	p.G360C heterozygote	-
		M	35.9%	31.0	11.5%	No mutation		-
		M	12.1%	30.4	14.7%	No mutation		-
		M	23.5%	13.9	16.2%	No mutation		-
C[a]	75	M	43.5%	10.6	56.9%	c.196G>C	p.E66Q	-
		F	35.8%	13.1	76.6%	No mutation		
	Normal Range		5.87±3.04 (mmol/L/h)	5.01±1.56	95.13±30.3 (nmol/mg/h)			0.17±0.13 (μg/mg Creatinine)

Genomic mutation nomenclature is based on GenBank NC_000023.9.

cDNA mutation nomenclature is based on GenBank NM_000169.1 with +1 corresponding to the A of the ATG translation initiation codon (www.hgvs.org/mutnomen). The initiation codon is codon 1.

[a]Group-A and -B newborns had <5 and 5 to 30% of normal mean leukocyte α-Gal A activity, respectively. Group-C newborns had >30% of normal mean leukocyte α-Gal A activity, and/or β-Gal A/α-Gal A ratio >10 for males, >20 for females.

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[b] g.9331G>A = c.639+919G>A = IVS4+919G>A; a previously reported cryptic splice variant [Ishii et al., 2002].

[c] Alternative splicing due to the IVS4+919G>A mutation results in a markedly reduced level of the normal α-Gal A glycoprotein.

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Table 2

In vitro expression and stability of wild-type and mutant α-Gal A constructs

		Stability at pH 4.6[b]	
Expressed Mutant Enzymes	**Activity % of WT**	**1hr**	**3hr**
WT	100%	90%	61%
p.H46P	36%	65%	23%
p.E66Q	52%	48%	13%
p.L120V	42%	73%	34%
p.I219T	46%	94%	77%
p.R356Q	15%	65%	23%
p.F383L[a]	35%	98%	78%
p.G360C	6%	66%	13%
p.D93N[a]	0%	-	-
p. C12_L14del	4%	-	-

Novel mutations in bold type

[a]p.D93N and p.F383L mutant proteins were previously reported [Stenson et al., 2003]

[b]Percent of initial activity