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First pilot newborn screening for four lysosomal storage diseases in an Italian region: Identification and analysis of a putative causative mutation in the GBA gene

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ABSTRACT

We report the first newborn screening pilot study in an Italian region for four lysosomal disorders including Pompe disease, Gaucher disease, Fabry disease and mucopolysaccharidosis type 1.
The screening has been performed using enzymatic assay on Dry Blood Spot on filter paper. A total of 3403 newborns were screened. One newborn showed a reduction of β-glucosidase activity in leucocytes. Molecular analysis revealed a status of compound heterozygous for the panethnic mutation N370S and for the sequence variation E388K, not yet correlated to Gaucher disease onset. The functional consequences of the E388K replacement on β-glucosidase activity were evaluated by in vitro expression, showing that the mutant protein retained 48% of wild type activity. Structural modeling predicted that the E388K replacement, localized to a surface of the enzyme, would change the local charges distribution which, in the native protein, displays an overwhelming presence of negative charges. However, the newborn, and a 4 year old sister showing the same genomic alterations, are currently asymptomatic.
This pilot newborn screening for lysosomal diseases appears to be feasible and affordable to be extended to large populations. Moreover other lysosomal diseases for which a therapy is available or will be available, could be included in the screening.

1. Introduction

Lysosomal storage diseases (LSDs) are a large group of more than 50 different inherited metabolic disorders which, in the great majority of cases, result from the lack or impaired function of specific acid hydrolases and, in few cases, of non-enzymatic lysosomal proteins involved in lysosomal biogenesis. To date, worldwide epidemiological data on LSDs are not available or are limited to distinct populations. As a group, overall incidence of LSDs is estimated at around 1:5000–1:7700 [1,2].

Symptoms in most LSD patients arise in the first few years of life. Mental retardation, joint weakness, hepatosplenomegaly, renal impairment and cardiovascular and respiratory pathology are commonly present, with a progressive severity. The need of frequent hospitalization and surgical intervention makes LSDs not only crippling disorders, but also an onerous expense for the health care system.

Although for some of these diseases, an enzyme-replacement therapy (ERT) is available, the initiation of treatment before symptoms are present is essential to improve the long-term outcome. Unfortunately, diagnosis is often delayed because of non-specific symptoms that frequently arise when organ damage is already irreversibly severe. For this reason, many newborn screening programs have been recently developed [2–6], by using several analytical techniques: enzyme assay, tandem mass spectrometry or immune-quantification.

We report the first enzyme assay newborn screening in Italy which involved the population of Umbria region. Between January 2010 and June 2012 over three thousand consecutive newborns were screened for 4 lysosomal disorders selected from those for which ERT is in clinical practice [7–10]: Pompe disease, also known as glycogen storage disease type II (GSD II; OMIM ID: 232300), Gaucher disease (GD; OMIM ID: 230800), Fabry disease (FD; OMIM ID: 301500) and mucopolysaccharidosis type

Abbreviations: LSD, lysosomal storage disease; ERT, enzyme replacement therapy; DBS, dried blood spots; GBA, acid β-glucosidase; GAA, acid α-glucosidase; GLA, acid α-galactosidase; GD, Gaucher disease; IDUA, α-L-iduronidase; MPS, mucopolysaccharidosis.

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1 (MPSI; OMIM ID: 607014; 607015), caused by the genetic deficiency of acid α-glucosidase (GAA, EC 3.2.1.20), acid β-glucosidase (GBA, EC 3.2.1.45), acid α-galactosidase (GLA, EC 3.2.1.22) and α-L-iduronidase (IDUA, EC 3.2.1.76), respectively.

Over a total of 37 retested newborns, pathological reduction of GBA activity was confirmed in leucocytes in one case. Molecular analysis revealed a status of compound heterozygous for the well known mutation p.N409S (N370S) and for the controversial variation c.1279G>A (E388K), found as rare allele in several series [11–14], but not yet univocally correlated to GD onset.

2. Materials and methods

2.1. Newborn screening

One thousand seven hundred and one female and 1702 male consecutive newborns from the Umbria area of central Italy were screened for acid α-glucosidase, acid β-glucosidase, acid α-galactosidase and α-L-iduronidase by using dried blood spots (DBS) on filter paper collected at age 2 days. All the samples were dried at room temperature for 4 h, stored at 4 °C in sealed plastic bags and analyzed within 15 days.

2.2. Ethical approval

The project was approved by the Umbria Regional Committee, and consent was obtained by parents for all the screened diseases. The family members of the newborn with reduction of GBA activity were counseled and were offered testing and medical evaluations.

2.3. Enzyme assays

Blood-spot activities were determined using a fully automated technique described elsewhere with the regular Guthrie card and commercially available fluorescent substrates [6,15]. Infants with activities<cut-off were considered positive and a second blood spot was requested and assayed. The established cut-off values were the 35% α-glucosidase normal median activity (normal median activity = 25 nmol/h/ml blood), 25% β-glucosidase normal median activity (24.5 nmol/h/ml), 25% α-galactosidase normal median activity (33.1 nmol/h/ml) and 25% α-L-iduronidase normal median activity (8.2 nmol/h/ml).

Acid α-galactosidase enzymatic activity was measured in both male and female newborns since two third of the female carriers can be identify using this technique [16,17].

In each analysis, DBS samples obtained from patients and healthy individuals were used as controls.

Retested newborns with confirmed blood spot activities<cut-off were recalled and tested with the conventional assay on leucocyte or lymphocytes.

GBA activity in white blood cells was determined using 4-MU-β-Glucopyranoside as a substrate in the presence of sodium deoxytaurocholate according to Raghavan et al. [18]. Plasmatic chitotriosidase activity was determined using the fluorogenic substrate 4-MU-β-D-N,N′,N″-triacetylchitotrioside.

2.4. Mutation nomenclature

Mutations are described as recommended, considering nucleotide +1 the A of the first ATG translation initiation codon [19, http://www.hgvs.org/mutnomen/]. Nucleotide numbers are derived from the *GBA* cDNA (GenBank ID: NM_000157.1). Traditional amino acid residue numbering (reference sequence AAC63056.1) has nevertheless also been provided in parentheses.

2.5. DNA amplification and sequencing

Genomic DNA was isolated from purified leucocytes with GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich). The exonic and most intronic sequences of the *GBA* gene were amplified by PCR using primers designed to amplify selectively the gene as described by Koprivica et al. [20]. PCR products were purified with the Qiagen Purification Kit (Qiagen GmbH, Hilden, Germany) and sequenced in the forward and reverse direction. Cycle sequencing was performed with the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) following the manufacturer's instructions. Putative mutations were confirmed by sequencing duplicate PCR products and by DNA analysis from parents.

2.6. Site-directed mutagenesis

The c.1279G>A (E388K) mutation was introduced in the wild type full length GBA cDNA cloned in pcDNA3 by PCR-based site-directed mutagenesis by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX, USA) according to the manufacturer's instructions. The following oligonucleotides were used: GauE388KFw 5′ ccttgccctgaaccc**a**aaggaggacccaattgg3′ and GauE388KRev 5′ccaattgggt cctcctt**t**ggggttcagggcaagg3′. PCR products were digested with 20 units of DnpI for 1 h at 37 °C and transformed in *Escherichia coli*. Each clone was completely sequenced to confirm that no other mutation was introduced by the mutagenesis procedure.

2.7. Cell culture and transient transfection

Hek293 cells were grown on monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 50 μg/ml penicillin/streptomycin (Gibco, Paisley, UK) and transfected with the wild type and mutant constructs using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Each construct was transfected in triplicate for three independent experiments. Cells were harvested after 72 h and assayed for acid β-glucosidase activity using the fluorogenic substrate 4-methylumbelliferyl-D-glucopyranoside (Sigma, St. Louis, MO, USA) in the presence of sodium deoxytaurocholate [18]. Protein concentration of the samples was determined by the Lowry method.

2.8. Molecular modeling studies

In order to analyse at a molecular level the effects of the amino acid replacement E388K, we carried over a visual inspection of the GBA three dimensional structure (PDB code: 2V3D) [21], using the program Coot [22]. Surface potential was calculated by the program DelPhi [23].

3. Results

3.1. Enzymatic assays

Three thousand four hundred and three newborns were screened for GAA, GBA, GLA and IDUA activities in blood spots; enzyme levels below 25% of the average control activities were founded in 12 (0.35%), 8 (0.23%), 4 (0.12%) and 13 (0.38%) neonates, respectively. A second blood spot was obtained from all 37 neonates. When retested, 3/12 neonates showed low GAA, 4/8 low GBA and 3/13 low IDUA activities. The remaining 27 neonates presented higher activities. Another whole blood sample (2 ml) was obtained from all 10 positive neonates, and enzyme activities were assayed in purified leucocytes (GBA and IDUA) or lymphocytes (GAA). A decreased level of GBA activity was confirmed only for one neonate (2.81 nmol/h/mg protein − normal mean: 14.1 ± 5 nmol/h/mg protein). Chitotriosidase activity in this newborn was 80 nmol/h/ml.

Levels of plasma chitotriosidase were evaluated in 280 newborns. Apart from 16 individuals showing the expected complete absence of