The Arg482His Mutation in the -Galactosidase Gene Is Responsible for a High Frequency of GM1 Gangliosidosis Carriers in a Cypriot

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The Arg482His Mutation in the β-Galactosidase Gene Is Responsible for a High Frequency of GM1 Gangliosidosis Carriers in a Cypriot Village

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ABSTRACT

GM1 gangliosidosis is a lysosomal storage disorder caused by deficiency of β-galactosidase. It is mainly characterized by progressive neurodegeneration, and in its most severe infantile form, it leads to death before the age of 4. The GLB1 gene gives rise to two alternatively spliced mRNAs that encode the β-galactosidase and the elastin binding protein (EBP). The diagnosis of two patients with the infantile form of GM1 gangliosidosis and 11 carriers in a small mountainous village in Cyprus prompted us to carry out a study in order to establish the frequency of carriers in the village and identify the mutations involved. Carrier detection was initially based on the measurement of β-galactosidase activity in leucocytes. Among 85 random samples from the village, 10 were classified as carriers. Sequencing of the GLB1 gene in a Cypriot patient identified the missense mutation c.1445G>A (p.Arg482His) in the homozygous state. Seven of the 10 carriers identified using the enzyme assay were found to carry the same mutation by *Nsp*I restriction enzyme analysis. The three individuals who were negative for the c.1445G>A had borderline enzyme results and were probably wrongly classified as carriers. The frequency of GM1 gangliosidosis carriers in this village is approximately 8% (1:12). Western blot analysis showed a marked decrease of the 64-kDa mature form of the enzyme protein and a similar reduction of the 67-kDa EBP. Our results indicate that the c.1445G>A mutation, which appears to be responsible for all GM1 gangliosidosis alleles in this Cypriot village, affects protein conformation.

INTRODUCTION

THE GM1 GANGLIOSIDOSIS (MIM 230500) is an inborn error of metabolism caused by the deficiency of a specific lysosomal hydrolase, β-galactosidase (E.C 3.2.1.23), which catalyzes the hydrolysis of terminal β-galactose linkages in ganglioside GM1 and other carbohydrate-containing compounds.

The disease has been classified into three clinical forms: type I (infantile), type II (late infantile/juvenile), and type III (adult) (Suzuki *et al.*, 2001). The most severe infantile form is characterized by psychomotor regression by the age of 6 months, facial and skeletal abnormalities, visceromegaly, and cherry-red spot. Patients rarely survive for more than a few years. Deficiency of β-galactosidase can also result in Morquio disease type B (MIM 253010), a characteristic mucopolysaccharidosis free of neurological symptoms (Callahan, 1999; Suzuki *et al.*, 2001). The incidence of GM1 gangliosidosis in most populations varies from 1:100,000 to 1:320,000 live births (Severine *et al.*, 1999).

The β-galactosidase gene (GLB1), mapped on chromosome 3 (3p21.33), has been cloned and characterized (Oshima *et al.*, 1988; Morreau *et al.*, 1989). The gene contains 16 exons and encodes a precursor protein of approximately 70-kDa, which is co-translationally modified into a glycosylated form of 85-kDa and processed in lysosomes into a 64-kDa mature enzyme (d'Azzo *et al.*, 1982; Nanba *et al.*, 1988).

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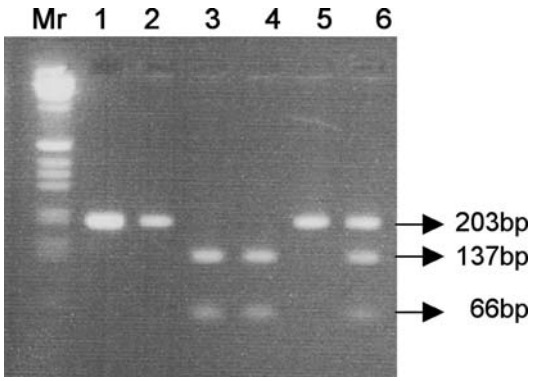

FIG. 1. Restriction analysis of genomic DNA, for the identification of the c.1445G>A mutation. A 203-bp fragment was PCR-amplified, digested with *Nsp*I, electrophoresed, and stained with ethidium bromide. Lane 6, heterozygous; lanes 3 and 4, homozygous; lanes 1, 2, and 5, normal controls. Mr, marker.

The GLB1 gene gives rise to two alternatively spliced mRNAs: a transcript of 2.5 kb, encoding the lysosomal enzyme, and a transcript of 2.0kb, encoding the elastin binding protein (EBP), which is localized on the cell surface (Hinek, 1996; Privitera *et al.*, 1998). Both the β-galactosidase and the EBP form complexes with protective protein cathepsin A and alpha neuraminidase.

A total of 49 mutations have been reported ⟨http://uwcmm/1s.uwcm.ac.uk/uwcm/mg/search/119987.html⟩. Five common mutations have been correlated with different clinical phenotypes: The Arg482His mutation detected in Italian patients correlated with infantile GM1 gangliosidosis and so did the mutation Arg208Cys detected in American patients. The Arg201Cys mutation detected in Japanese patients correlated with juvenile GM1 gangliosidosis, whereas the Ile51Thr mutation, also found in Japanese patients, correlated with adult GM1 gangliosidosis. The Trp273Leu mutation found in Caucasian patients correlated with Morquio B disease (Suzuki *et al.*, 2001).

Two patients with the classical, infantile form of GM1 gangliosidosis were diagnosed in two different families in Cyprus in 1992. Both cases came from the village of Pelendri, which is located in a mountainous area of the island. The fathers of the patients are first cousins. Prior to this study, 29 persons from this village were examined for carrier status of the disease, and 11 of them were found to be carriers. This relatively high number of carriers prompted us to initiate an epidemiological study in order to establish the frequency of the mutant allele in the village. A total of 104 volunteers were screened for carrier status. The screening was initially based on the measurement of β-galactosidase activity in leucocytes. Molecular studies were performed in order to identify the mutations involved, and expression studies followed on the identified mutation.

MATERIALS AND METHODS

Subjects

Patient 1 was a boy, the first child of young, healthy, unrelated parents. He had coarse dysmorphic features, psychomotor delay, and edema of the eyes, limbs, and scrotum. At 9 months of age, he could not concentrate or fix his eyes and did not reach out for objects. There were no focal neurological findings. He also had mild hepatosplenomegaly, and hypertonia of upper and lower limbs. The diagnosis was established by measuring β-galactosidase activity in leucocytes, which was found to be 5 nmol/hr/mg protein (normal, 163–378 nmol/hr/mg protein). The family history revealed that the mother's grandmother gave birth to six children, five of whom died at the age of 1–2 years.

Patient 2 was also a boy, the first child of the family. The parents are third cousins. He presented with hypertonia since birth and developed edema of the face, eyes, and lower limbs. His psychomotor development was delayed, and he had a mildly coarse face. The diagnosis was established by measuring β-galactosidase activity in leucocytes, which was found to be 4 nmol/hr/mg protein (normal, 163–378 nmol/hr/mg protein).

The aborted affected fetus of a couple who had undergone prenatal diagnosis for GM1 gangliosidosis at 12 weeks of pregnancy was used to establish a fibroblast cell line. The β-galactosidase activity in the chorionic villi of this fetus was 1.5 nmol/hr/mg protein (normal, 116–893 nmol/hr/mg protein).

In order to establish a reference range for leucocyte β-galactosidase activity in the Cypriot population, 102 apparently healthy individuals, originating from various districts of the island (52 men and 50 women, mean age 36 years old), were examined after giving their informed consent.

In order to establish the frequency of the mutant allele, a total of 104 volunteers from the village of Pelendri were examined after giving their informed consent. Of these, 54 were men and 50 were women, aged 20–80 (mean age, 38 years old). Samples from the volunteers were collected on three different occasions during organized visits to the village. A talk on the subject by a geneticist preceeded the blood collection. All volunteers were asked to fill in a questionnaire in order to ascertain whether they were related to known patients or carriers. Subjects with a blood relation who was either affected or a carrier were considered as having a positive family history. If more than one member of an extended family gave blood, only the first sample was considered random; the rest were designated as "non-random."

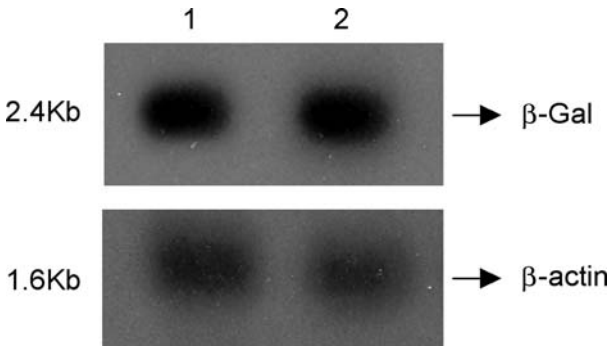

FIG. 2. Northern blot analysis of cultured skin fibroblasts from normal control (lane 1) and GM1 gangliosidosis fetus [c.1445G>A]+[c.1445G>A] (lane 2). *Upper panel,* 0.8-kb human β-gal cDNA. *Lower panel,* the same blot hybridized with human β-actin cDNA, to confirm RNA quality and relative loading.

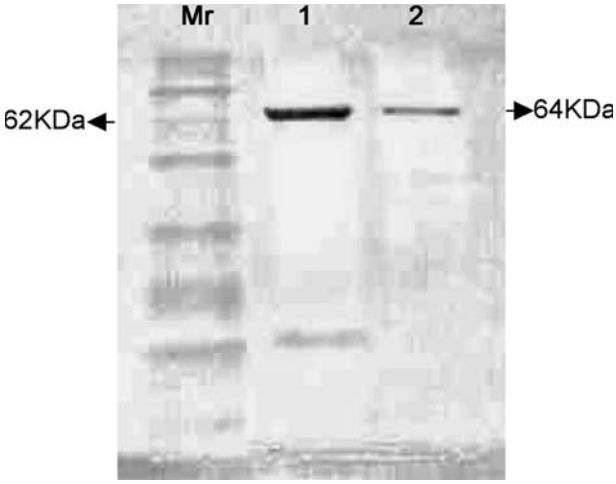

FIG. 3. Western blot analysis of cultured skin fibroblasts for the detection of β-gal protein from a GM1 gangliosidosis fetus [c.1445G>A]+[c.1445G>A] (lane 2) and normal control (lane 1). Mr, marker (P7708S Biolabs).

Biochemical analysis

For enzymatic assays, the cells (leucocytes, fibroblasts, or transfected COS-1 cells) were lysed by sonication in water. The activity of β-galactosidase was measured using the 4-methylumbelliferyl-β-D-galactopyranoside substrate, according to Galjaard (1980). Protein was determined as described by Smith. (1985), using bovine serum albumin as a standard (Sigma P-0914).

PCR amplification and sequencing

Genomic DNA was extracted from peripheral blood using the method of Miller *et al.* (1988). PCR reactions for all 16 exons of the β-galactosidase gene were carried out in 50-μl volumes containing 50 ng of genomic DNA, 50 pmol of each oligonucleotide, 5.0 U of Tag polymerase (Gibco), 2.5 U pfu turbo DNA polymerase (Stratagene), and 100 mM of each of dCTP, dGTP, dTTP, and dATP in 2 M Tris-HCl (pH 8.8), 1 M ammonium sulphate (Sigma A-4418), 1 M $MgCl_2$ (Sigma M-1028), 100% 2-mercaptoethanol (Sigma M-3148), 10 mM EDTA pH 8.0 (Sigma E-7889), 10 mg/ml BSA (Amersham Pharmacia cat. no. 27-8915). The amplification procedure for every fragment started with a 30-sec denaturation step at 94°C, followed by 30 cycles of 20-sec denaturation, 20 sec at the annealing temperatures and a 20-sec extension at 70°C. After the last cycle, the samples were incubated for 10 min at 70°C for the final extension. The annealing time was the melting time of the oligonucleotide primers minus 5°C. The oligonucleotides used for PCR reactions are as published by Georgiou *et al.* (2004). Control PCR reactions in which no template DNA was added were included during each set of PCR reactions.

PCR products were run on an agarose gel (1.5–2.0% depending on PCR product size), excised and purified using QIAquick Gel Extraction Kit (Qiagen). Approximate 30 ng of purified amplification product were used in sequencing reactions. Both strands were sequenced with the same primers used for PCR amplification. DNA variation numbering was based on GenBank NM-00404.1 with +1 as A of the ATG start codon (consensus nomenclature of mutations according to Dunnen and Antonarakis [2001]).

Restriction enzyme analysis

To examine the G>A transition in codon 482 (Arg482His), a 10-μl sample containing the 203-bp PCR product from exon 14 was digested with 10 units of *Nsp*I for 1 hr at 37°C and run on a 3% agarose gel.

Cell cultures

Human skin fibroblasts from normal controls and from an abortus affected with GM1 gangliosidosis were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (10 IU/ml), and streptomycin (10 IU/ml).

Northern blot analysis

Total RNA was isolated from cultured skin fibroblasts from a normal control and from the GM1 gangliosidosis abortus, using the Rneasy Midi kit (QIAGEN). The RNA was separated on a 1% agarose gel containing 0.6 M formaldehyde. After electrophoresis, the RNA was transferred to a Zeta-probe membrane (Bio-Rad) and immobilized by baking at 80°C for 2 hr. A 0.8-kb length of human β-gal cDNA was used as a probe. Pre-hybridization and hybridization of the blot was performed using ExpressHyp mix (CloneTech). After hybridization, subsequent washings were performed according to the manufacturer's protocol. The blot was re-probed with radiolabeled β-actin cDNA, after stripping the filter by boiling it for 10 min in 0.05% SDS.

Western blot analysis

For Western blotting, human skin fibroblasts from a normal control and from the GM1 gangliosidosis abortus were harvested by scraping in PBS and lysed by sonication. Cell lysates

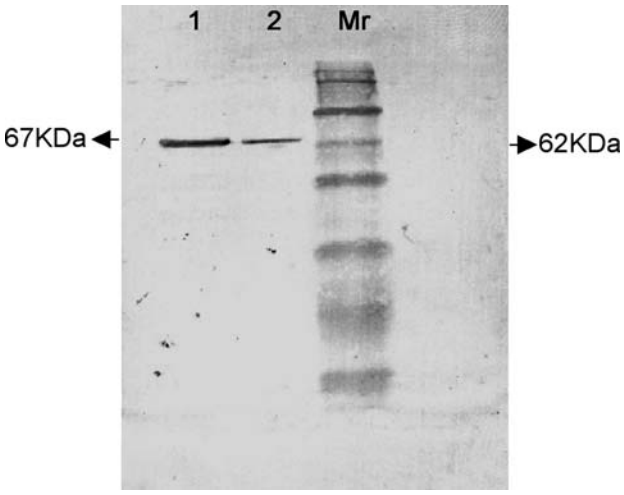

FIG. 4. Western blot analysis of cultured skin fibroblasts for the detection of elastin binding protein (EBP) in a GM1 gangliosidosis fetus [c.1445G>A]+[c.1445G>A] (lane 2) and normal control (lane 1). Mr, marker (P7708S Biolabs).

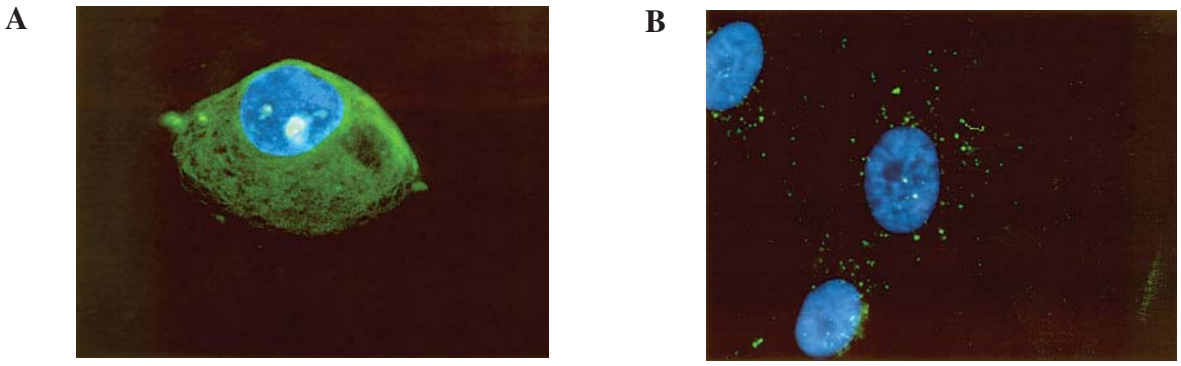

FIG. 5. Immunofluorescence detection of β-galactosidase in fibroblasts: normal control (**A**) and GM1 gangliosidosis fetus (**B**) [c.1445G>A]+[c.1445G>A]. (Fig. 5 in color can be viewed in the online version of this issue, which is available at www.liebertpub.com)

(20 μg of protein) were subjected to SDS-PAGE electrophoresis and transferred to a nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were incubated for 16 hr with anti–β-gal native antibody (prepared in Dr. d'Azzo's lab). The anti-β-galactosidase antibody was raised against a purified placental preparation of β-galactosidase, and it binds the precursor and the mature form of the enzyme. For the detection of the elastin binding protein, the membranes were incubated for 16 hr with anti:Vs ALF1 antibody (Malvagia *et al.*, 2004). An anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma A-9919) was then added, and the band was revealed using an AP conjugate substrate kit (Bio-Rad, Hercules, CA).

Expression studies in COS-1 cells

In vitro mutagenesis of human β-galactosidase cDNA was carried out using the method of Higuchi *et al.* (1988) to construct the mutated vectors. The mammalian expression vector pcDx was used as a PCR template.

The primers used for the c.1445G>A (p.Arg482His) were as follows:

Lsense:	5′-CATTCCTGAATGTGGGGCCCTGC-3′
Ex14anti:	5′-CATAGTTCACATGTCCCATGTTC-3′
Ex14sense:	5′-GAACATGGGACATGTGAACTATG-3′
Lanti:	5′-TCCCTCAAAGACACAGGCTTTCATC-3′

In order to introduce the Arg482His mutation into the full-length cDNA, fragments (~1 Kb) containing the mutation of interest were excised from PCR products by using two restriction endonucleases, each of which has only one recognition site in the β-galactosidase cDNA (*Dra* III and *Bst*X1). After electrophoresis in 1% agarose gels, the DNA fragments were purified with QIAquick Gel Extraction Kit (Qiagen). The pcDx vector that contained the wild-type β-galactosidase cDNA was restricted at two unique endonuclease sites to excise the corresponding wild-type fragment. The fragment carrying the mutation was then ligated into the plasmids.

Normal and mutant vectors were transfected into African green monkey kidney cells, as described by Caciotti *et al.* (2003). Transfected COS-1 cells were cultured, and the activity of the β-galactosidase enzyme was assayed at 48 hr post-transfection.

Immunofluorescence

Immunolocalization of β-galactosidase was performed in human skin fibroblasts from a normal control and from the GM1 gangliosidosis fetus. Cells were grown on glass coverslips for 48 hr and were subsequently fixed for 10 min in 3% paraformaldehyde, in 0.1 M Na-Phosphate buffer pH 7.3, and permeabilized with 100% cold methanol at −20°C for 30 min. The fixed cells were then washed in PBS with 0.5% BSA and 0.15 M glycine, and incubated overnight with the primary antibody (prepared in Dr. d'Azzo's lab), diluted 1:100. The secondary antibody, a green fluorescein-labeled goat-(anti-rabbit) IgG diluted 1:100, was then added for 1 hr. The cells were washed three times with PBS containing 0.5 M BSA and 0.15 M glycine, and the nuclei were stained by adding 10 μl of DAPI for 1 min. The cells were washed again three times, and they were examined under a fluorescence microscope.

TABLE 1. β-GALACTOSIDASE ACTIVITY IN COS-1 CELLS TRANSFECTED WITH MUTANT β-GALACTOSIDASE

	β-Galactosidase activity nmol/hr/mg protein	*Percentage of wild-type*
COS-1 +wild type-pcDx	765	100
COS-1 +Arg482His-pcDx	105	0
COS-1 not transfected	105	—

Each experiment was performed in triplicate, and the values (nmol/hr/mg protein) are the mean values.

Table 2. β-Galactosidase Activity in Leucocytes of Normal Subjects, Definite Carriers, and Possible Carriers of GM1 Gangliosidosis

	Enzyme activity nmol/hr/mg protein	*Mean*	*SD*
Control population, $n = 100$	169–347	252.8	39.0
Definite carriers[a] positive for the Arg482 His mutation, $n = 15$	95–160	116.1	25.9
Possible carriers negative for the Arg482His mutation, $n = 3$	159, 173, 175	—	—

[a]In this group, we included four obligate carriers, the parents of patients 1 and 2.

RESULTS

The enzyme values of the 102 healthy volunteers were analyzed using the "box plot" method in order to identify any outliers. Two outliers were identified and discarded. The distribution of the remaining 100 values was tested for normality using the Kolmogorov-Smirnov test and found to be normal. This allowed us to use the mean ± 1.96 SD as the reference range, which is 175–331 nmol/hr/mg protein. Subjects with an enzyme activity lower than 175 nmol/hr/mg protein were designated as carriers of GM1 gangliosidosis. According to this criterion, 10 carriers were identified among 85 random samples, two among nine "non-random samples," and two among 10 samples from persons with a family history.

Sequencing of the genomic DNA of patient 1 resulted in the identification of a previously reported missense mutation, c.1445G>A (Arg482His). The patient was homozygous for the mutation. The mutation was confirmed by restriction analysis using the *Nsp*I enzyme, which creates a natural restriction site. The 203-bp PCR product is digested to 137-bp and 66-bp fragments (Fig. 1). The parents of patient 2 were shown by restriction analysis to be heterozygous for this mutation. The DNA samples from the volunteers of the village were also screened for the Arg482His mutation. Eleven out of the 14 carriers identified in the biochemical screening were positive and three were negative for this mutation. Subjects designated as normal in the biochemical screening were negative for the mutation.

The GM1 gangliosidosis fetus was found to be homozygous for the Arg482His mutation. Northern blot analysis in skin fibroblasts from this fetus showed that the β-galactosidase mRNA was present at levels similar to those in normal fibroblasts (Fig. 2). Western blots of total homogenates of cultured fibroblasts were analyzed with affinity-purified anti–β-gal antibodies. The amount of the 64-kDa mature form of the β-galactosidase enzyme is markedly reduced in the GM1 gangliosidosis fetus compared to the wild-type control (Fig. 3). When the same blot was analyzed with an anti-EBP antibody, a similar reduction in the quantity of the elastin binding protein was detected in the fetus (Fig. 4). Immunocytochemical localization of β-galactosidase in skin fibroblasts from the GM1 gangliosidosis fetus shows that the level of the immunodetectable enzyme is very low compared to a normal control (Fig. 5).

The expression studies in COS-1 cells revealed that the Arg482His mutation abolishes the activity of β-galactosidase. The activity of the transfected cells was found to be equal to the background activity of non-transfected cells (Table 1).

DISCUSSION

Carrier screening using the enzyme assay resulted in the identification of a total of 14 carriers among 104 individuals from the village of Pelendri. Of the 104 samples, only 85 were random, and of these 10 were found to be carriers. Using the PCR-based test for the mutation, 11 of 14 samples characterized as carriers biochemically were found to have the Arg482His missense mutation. Of the 10 carriers found in the random group, only seven carried this mutation. The three samples that were negative for the Arg482His mutation had equivocal enzyme levels (Table 2) and were probably incorrectly classified as carriers by the biochemical test, although the presence of a second mutation has not been excluded. Screening for carriers of any genetic disorder using a biochemical test has the advantage that it can potentially pick up all carriers irrespective of the mutation involved. It does, however, have the disadvantage that there is usually an overlap between control and carrier ranges. This situation is evident in the results of our study.

Based on the results of the random group only, we conclude that there is a high frequency of GM1 gangliosidosis carriers in the village of Pelendri, about 8.3% (95% confidence interval, 2.43–14.17%). This high frequency is most probably due to a founder effect followed by genetic drift and/or inbreeding. Due to its location in the mountains, the village was most probably isolated in the past when means of transport were underdeveloped. A family originating from a village within 30 km of Pelendri was also found to carry the Arg482His mutation. Thus, we might expect to find this allele among people originating from a wider area around Pelendri. The population of Pelendri today is around 1500, but a much larger number of people originating from this village now live all over the island. Increased frequency of GM1 gangliosidosis has been reported in other populations. In Malta, GM1 gangliosidosis is the most common inherited metabolic disorder, affecting one individual per 3700 live births, with a carrier frequency of 1:30 (Lenicker *et al.*, 1997). Increased incidence of GM1 gangliosidosis is also found in South Brazil, where it reaches a value of 1:1700 live births in Porto Alegre City (Severini *et al.*, 1999; Silva *et al.*, 1999).

The mutation identified in our population was first reported in 1991 by Oshima *et al.* in two Italian patients with Morquio B disease; they were compound heterozygotes for the Arg482His and Trp273Leu mutation. The Arg482His amino acid substitution was also reported in 1992 in one Italian patient who was homozygous and in six unrelated Italian patients

who were heterozygous, all of Sicilian origin (Mosna *et al.*, 1992). More recently, a GM1 gangliosidosis patient from Campania, South Italy, who had cardiac involvement was reported to be heterozygous for Arg482His (Morrone *et al.*, 2000). The Arg482His was also identified in two Australian patients of Maltese origin (Caciotti *et al.*, 2005). The evidence so far thus indicates that the Arg482His mutation is common in the Mediterranean area. It is interesting that it is the only mutation in the β-galactosidase gene found on the islands of Cyprus and Malta. The two islands have historic connections, especially through the Order of St. John, which had strongholds in both islands. It is tempting to speculate that the mutation was spread around the Mediterranean by the crusaders and the knights of St. John. A more detailed study into the polymorphisms associated with this mutation in the different Mediterranean populations might prove rewarding.

Our study has shown that the Arg482His mutation results in mRNA of normal size and quantity. These results agree with those of Mosna *et al.* (1992). The amount of the 64-kDa mature form of the protein is substantially decreased in the homozygote fetus as shown by Western blot analysis. To our knowledge, this is the first time that Western blot studies were preformed for this mutation. The immunofluorescence studies, also performed for the first time, showed absence of β-galactosidase from its normal site. Based on these results, it would be reasonable to suggest that the Arg482His mutation alters the folding and/or affects the stability of the mature β-galactosidase. More direct evidence supporting this hypothesis needs to be obtained. A comparison of the sequence at the site of the mutation with that in other species (using the Clustal W program, European Bioinformatics Institute) has shown that the Arg482 is evolutionary conserved, indicating its importance for enzyme function. When the Arg482His mutation was expressed in COS-1 cells, the enzyme activity did not increase above the endogenous level found in non-transfected COS-1 cells. These results agree with those of Oshima *et al.* (1991) from expression studies of the Arg482His mutation in transformed human fibroblasts.

The amount of EBP in the homozygote fetus was also decreased, as shown by Western blot analysis. This is expected since EBP arises by alternative splicing of the primary Gal transcript, whereby exons 3, 4, and 6 are skipped, and there is a frameshift in exon 5. The reading frame is restored at the start of exon 7. Since the Arg482His mutation is located in exon 14, the conformation of EBP is expected to be affected also.

In conclusion, this study has revealed a high frequency of the Arg482His mutation (8.3%) among the people of the village of Pelendri in Cyprus, and it has shed some light on the possible effect of this mutation on enzyme structure and function.

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