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MUTATION IN BRIEF

Four Novel Mutations in Patients From the Middle East With the Infantile Form of GM1-gangliosidosis

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GM_1-gangliosidosis is a lysosomal storage disorder caused by a 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 four. We have performed molecular analysis of five patients with the infantile form of GM1-gangliosidosis originating from the Middle East (two from Saudi Arabia and three from the United Arab Emirates). We have identified four novel mutations and one previously reported mutation in the GLB1 gene. The first novel mutation found in the homoallelic state in a patient from Saudi Arabia, is a c.171C>G transversion in exon 2 which creates a premature stop codon. Northern blot analysis in fibroblasts from the patient showed no mRNA and expression studies in COS-1 cells showed complete absence of the 85kDa precursor protein and no catalytic activity. The second novel mutation is a splicing error in intron 2, c.245+1G>A. This mutation was found in the heteroallelic state in a patient from Saudi Arabia, the second mutation being the previously described c.145C>T mutation. The third novel mutation is a missense mutation in exon 4, c.451G>T, found in the homoallelic state in a patient from the United Arab Emirates. Expression studies of this mutation in COS-1 cells showed complete absence of the 85kDa precursor protein and no catalytic activity. The fourth novel mutation is a splicing mutation in intron 8, c.914+4A>G, found in the homoallelic state in two siblings from the United Arab Emirates. This study has revealed genetic heterogeneity of the β-galactosidase deficiency in the Arabic population. © 2004 Wiley-Liss, Inc.

KEY WORDS: GM1-gangliosidosis; GLB1; Middle East; United Arab Emirates; Saudi Arabia

INTRODUCTION

GM1-gangliosidosis (MIM# 230500) is an inborn error of metabolism caused by deficiency of a specific lysosomal hydrolase, β-galactosidase (E.C 3.2.1.23) which catalyses the hydrolysis of terminal β-galactose

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linkages in ganglioside GM1 and other carbohydrate-containing compounds. The disorder is mainly characterized by progressive neurodegeneration and has been classified into three clinical forms: infantile (type I), late infantile or juvenile (type 2) and adult or chronic (type 3) (Suzuki et al., 2001). The clinical manifestation of the most severe infantile form includes rapidly progressive CNS involvement, facial and skeletal abnormalities and visceromegaly. Patients rarely survive for more than a few years. Deficiency in β-galactosidase can also result in Morquio disease type B (MIM# 253010), a characteristic mucopolysaccharidosis free of neurological symptoms (Callahan et al., 1999; Suzuki et al., 2001). Furthermore, patients with infantile GM1-gangliosidosis who also have cardiac involvement have been shown to be deficient in both the β-galactosidase and the β-galactosidase related protein, also known as elastin binding protein, which results from the GLB1 gene by alternative splicing (Privitera et al., 1998; Morrone et al., 2000).

The β-galactosidase gene (GLB1) has been mapped on chromosome 3 (3p21.33) and has been cloned and characterized (Oshima et al., 1988; Morreau et al., 1989). The gene contains 16 exons and encodes a precursor protein approximately 70kDa which is co-translationally modified into a glycosylated form of 85kDa and processed in lysosomes into a 64kDa mature enzyme (D'Azzo et al., 1982; Nanba et al., 1988). A total of 48 mutations have been reported (http://uwcmm/1s.uwcm.ac.uk/uwcm/mg/search/119987.html). Of these, 38 are missense/non-sense mutations, three are splicing mutations, five are small insertions and two are duplications.

In this study we report four new mutations in five patients with the infantile form of GM1-gangliosidosis originating from the Middle East.

MATERIALS AND METHODS

Patients

Five patients with the infantile form of GM1-gangliosidosis from the Middle East were included in the present study, two from Saudi Arabia and three from the United Arab Emirates. Their diagnosis was based on the clinical history and confirmed by the measurements of β-galactosidase activity in leucocytes or fibroblasts.

Mutation Analysis

Genomic DNA was extracted from peripheral blood or skin fibroblasts of affected patients according to standard methods. All the 16 exons of the β-galactosidase gene and their flanking intronic regions were amplified by PCR and automated sequencing was carried out using the Perkin Elmer "Big Dye Terminator Cycle Sequencing Ready Reaction Kit" as described by the manufacturer. Electrophoresis and sequence analysis followed on an ABI PRISM 310 Genetic Analyser.

The oligonucleotides used for PCR reactions are as published by Silva et al. (1999) except for exons 1,2,6,14,15,16 which are the following:

Exon 1 sense : 5'-CCTGGGCGCCGACTGCAGAG-3'
Exon 1 antisense : 5'-CCTAGCAATGCCTCCCCGTA-3'
Exon 2 sense : 5'-AAGGTGGAAGTTCTCATGTG-3'
Exon 2 antisense : 5'-TGTTCAGGCCTAGGTGAGAG-3'
Exon 6 sense : 5'-AGGATCTCCTCATTTTTCCCT-3'
Exon 6 antisense : 5'-TAAGCTGCAATTTCTGTTAC-3'
Exon 14 sense : 5'-ACCCTCAATTCAATTGTCAT-3'
Exon 14 antisense : 5'-AACCTTAGTCTTGACAGTGT-3'
Exon 15 sense : 5'-GAATTCAAACCCTTCCCATG-3'
Exon 15 antisense : 5'-CAGCTCACTGTGCTCTGTTT-3'
Exon 16 sense : 5'-TGGGGTTGATGGTTCTCTGT-3'
Exon 16 antisense : 5'-CATGACAGGGAGGATCTG-3'

Northern Blot Analysis

Total RNA and polyA$^+$RNA were isolated from cultured fibroblasts using the Oligotex and RNAeasy RNA purification Kits (Qiagen) and Northern Blot analysis was performed. As a probe a 0.8 kb length human β-galactosidase cDNA was used. To compare the quantity and quality of different RNA samples, the Northern blot was also hybridised with a β-actin probe. The intensities of β-gal mRNA bands were normalized by calculating their ratios to the intensities of β-actin mRNA bands.

Expression Studies

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

For the c.171C>G mutation the primers used were:

3040 sense: 5'-TGCAGGTCGACTCTAGAGGATC-3'
T57X anti: 5'-GGCACACGGGACTAGTGAATGCTTC-3'
T57X sense: 5'-GAAGCATTCACTAGTCCCGTGTG-3'
H anti: 5'-TCGTGGACTCCATTGAGGGGTGAAGAG-3'

For the c.451G>T mutation the primers used were:

3040 sense: 5'-TGCAGGTCGACTCTAGAGGATC-3'
D151Y anti: 5'-GGTAATCTGGGTAGGAGGAGCGGAG-3'
D151Y sense: 5'-CTCCGCTCCTCCTACCCAGATTACC-3'
H anti: 5'-TCGTGGACTCCATTGAGGGGTGAAGAG-3'

The mutations of interest were excised from PCR products using two restriction endonucleases each of which has only one recognition site in the GLB1 cDNA. The restriction enzymes used were the XbaI (5'restriction site) and the DraIII (3' restriction site). The pcDX vector which contained the wild type GLB1 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, using the DNA Ligation kit ver.2 by Takara Biomedicals.

Normal and mutant vectors were transiently overexpressed into African green monkey kidney cells (COS-1) according to Sambrooks et al. (1989). Transfected COS-1 cells were cultured and 48 hours post transfection the activity of the β-galactosidase enzyme was assayed with the artificial 4-methylumbelliferyl beta-D-galactopyranoside substrate, as described by Caciotti et al. (2003).

For Western blotting transfected COS-1 cell lysates were used. The antibody was raised against a purified placental preparation of β-galactosidase and it binds the precursor and the mature form of the enzyme.

RESULTS AND DISCUSSION

In this study five patients from the Middle East with the infantile form of GM1-gangliosidosis, coming from four unrelated families, were studied at the molecular level. As far as we know, this is the first report of GLB1 mutations in the Arabic population. All ten alleles were characterized and five different mutations were found, four of which are novel. For all the patients, the 16 exons and their flanking regions were amplified and directly sequenced on both strands. The data of the five patients analysed including their geographic origin and their genotypes, are summarized in Table 1.

Table 1. β-galactosidase Mutations in GM1-gangliosidosis Patients

Patient	Sex	Ethnic origin	GLB1 Base change	Location exon/ intron	Amino acid change	Type of mutation	Activity of β-gal enzyme % of normal mean
Case 1	F	Saudi Arabia	[c.171C>G]* + [c.171C>G]*	Exon 2	p.Tyr57X	Nonsense	0.37 (SF)
Case 2	M	Saudi Arabia	[c.145C>T] + [c.245+1G>A]*	Exon 2 Intron 2	p.Arg49Cys	Missense Splicing	0.73 (SF)
Case 3	M	United Arab Emirates	[c.451G>T]* + [c.451G>T]*	Exon 4	p.Asp151Tyr	Missense	1.7 (L)
Case 4.1	M	United Arab Emirates	[c.914+4G>A]* + [c.914+4G>A]*	Intron 8		Splicing	1.27 (L)
Case 4.2	F	United Arab Emirates	[c.914+4G>A]* + [c.914+4G>A]*	Intron 8		Splicing	1.31 (SF)

DNA variation numbering based on GenBank NM_000404.1, with +1 as A of the ATG start codon. Introns based on genomic GenBank NT_022517.16. Consensus nomenclature of mutations according to Dunnen and Antonarakis (2000).
*Mutations that have not been previously reported. SF: skin fibroblasts L: leucocytes

Patient 1, a girl from Saudi Arabia, was found to be homozygous for a c.171C>G transversion which creates a premature stop codon. This mutation has not been previously described. As expected, Northen blot analysis showed no total and no mRNA (Fig. 1).

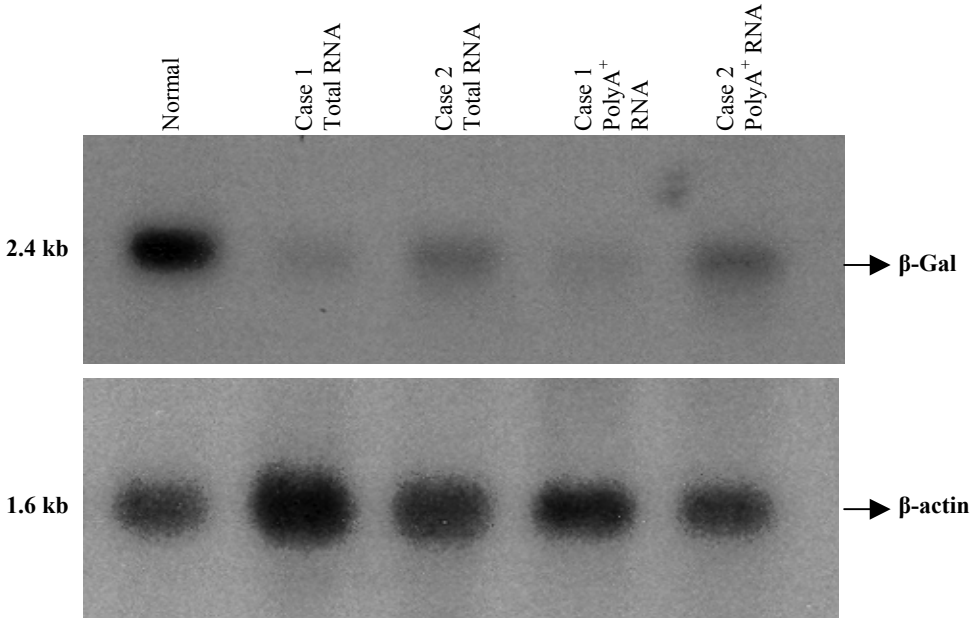

Figure 1. Northern blot analysis of cultured fibroblasts from case 1, [c.171C>G] + [c.171C>G] and case 2 [c.145C>T] + [c.245+1G>A]. Top, probe was a 0.8 kb human β-gal cDNA. The lower panel represents the same blot hybridised with human β-actin cDNA to confirm RNA quality and relative loading. The size of the two transcripts was calculated on the basis of the 0.24-9.5 kb RNA Ladder (Life technologies). Exposure time for both hybridisations was 24h.

The c.171C>G transversion was expressed in COS-1 cells. COS-1 cells transfected with the mutant vector showed absence of the 85kDa precursor protein (Fig. 2) and no catalytic activity (Table 2). The 64kDa mature protein is absent in all preparations including the positive control COS-1 cells transfected with normal β-gal vector. It was previously shown that in COS-1 cells transiently expressing human β-galactosidase, the 85kDa precursor is poorly processed into the mature 64kDa protein (Oshima et al., 1988; Morreau et al., 1989). The lack of processing is thought to be due to the transfection procedure and the fact that the amount of endogenous protective protein available in COS-1 cells is not sufficient to stabilize the bulk of over produced β-galactosidase molecules.

Figure 2. Western blot analysis of non transfected and transfected Cos-1 cells. Lane 1 (negative control) corresponds to non transfected COS-1 cells. Lane 2 (positive control) are COS-1 cells transfected with normal β-gal vector. Lane 3 corresponds to COS-1 cells transfected with mutated vector Arg482His. Lane 4 and 5 are COS-1 cells transfected with mutated vectors Tyr57 stop codon and Asp151Tyr respectively. Mr is the marker.

The novel splice mutation in intron 2, c.245+1G>A was found in the heteroallelic state in Patient 2, a boy from Saudi Arabia. It is known that mutations in the gt/ag oligonucleotides at the 5' and 3' splice sites interfere with normal splicing (Padgett et al., 1986). The second mutation in this patient is a missense mutation, c.145C>T (p.Arg49Cys) previously found in the heteroallelic form in a Japanese patient with infantile GM1-gangliosidosis (Nishimoto et al., 1991). Fibroblasts from this patient showed substantially decreased amounts of total and mRNA in the Northern blot (Fig. 1). In order to delineate the separate effects of these two mutations expression studies or patients homozygous for either mutation are needed.

Patient 3, a boy from the United Arab Emirates, was found to be homozygous for a G>T transition at nucleotide 451, which results in an aspartic acid to tyrosine substitution in codon 151. This mutation, which has not been described before, was expressed in COS-1 cells. Transfected COS-1 cells showed complete lack of the 85kDa precursor protein (Fig. 2) and no enzymatic activity (Table 2). COS-1 cells transfected with another missense mutation (c.1445G>A, p.Arg482His) were run at the same time and they showed normal amounts of the 85kDa precursor protein (Fig. 2). It could be hypothesised that the aspartic acid residue at position 151, which is highly conserved, is important for the stability of the enzyme and its substitution leads to early degradation. For another missense mutation only three residues away from our mutation (p.Arg148Ser), Zhang et al. (2000) found abundant amounts of the precursor in COS-1 cells. More detailed studies on the structure and processing of the β-galactosidase protein are needed in order to understand the impact of the various missense mutations on enzyme activity.

Table 2. β-galactosidase Activity in COS-1 cells Transfected with Mutant β-galactosidase Constructs

	β-galactosidase activity nmole/hr/mg protein	% of wild-type
COS-1 + wild type-pcDx	707	100
COS-1 + Tyr57 stop codon-pcDx	87	0.56
COS-1 + Asp151Tyr-pcDx	83	0
COS-1 not transfected	83	–

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

Patients 4.1 and 4.2 are siblings from the United Arab Emirates and they were found to be homozygous for a novel mutation, a transition of A>G in intron 8, c.914+4A>G which could affect the splicing process that normally removes the intron sequence from the primary RNA transcript. Unfortunately no skin fibroblasts were available to confirm this hypothesis by RNA studies. Consensus sequences for RNA splicing in higher eucaryotes is for the 5' splice site (donor site) GUA (or G) AGU and for the 3' splice site (acceptor site) C (or U) AG (Alberts et al., 1994). The parents, who are first cousins, were found to be carriers for this mutation. Since no other mutation was detected by screening the GLB1 gene in these patients we assume that this mutation is responsible for the disease.

The detection of five different mutations in four families studied indicates genetic heterogeneity of the β-galactosidase deficiency in the Arabic population. Molecular characterization of more GM1-gangliosidosis patients in the Arabic as well as other populations will most likely yield more new mutations and allow a better understanding of genotype-phenotype correlations.

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