Short Report

Phenotype determining alleles in GM1 gangliosidosis patients bearing novel \textit{GLB1} mutations


GM1 gangliosidosis manifests with progressive psychomotor deterioration and dysostosis of infantile, juvenile, or adult onset, caused by alterations in the structural gene coding for lysosomal acid \(\beta\)-galactosidase (\textit{GLB1}). In addition, allelic variants of this gene can result in Morquio B disease (MBD), a phenotype with dysostosis multiplex and entire lack of neurologic involvement. More than 100 sequence alterations in the \textit{GLB1} gene have been identified so far, but only few could be proven to be predictive for one of the GM1 gangliosidosis subtypes or MBD. We performed genotype analyses in 16 GM1 gangliosidosis patients of all phenotypes and detected 28 different genetic lesions. Among these, p.I55FfsX16, p.W65X, p.H112P, p.C127Y, p.W161X, p.I181K, p.C230R, p.W273X, p.R299VfsX5, p.A301V, p.F357L, p.K359KfsX23, p.L389P, p.D448V, p.D448GfsX8, and the intronic mutation IVS6-8A>G have not been published so far. Due to their occurrence in homozygous patients, four mutations could be correlated to a distinct GM1 gangliosidosis phenotype. Furthermore, the missense mutations from heteroallelic patients and three artificial nonsense mutations were characterized by overexpression in COS-1 cells, and the subcellular localization of the mutant proteins in fibroblasts was assessed. The phenotype specificity of 10 alleles can be proposed on the basis of our results and previous data.

Two lysosomal storage diseases, GM1 gangliosidosis (GM1; MIM# 230500) and Morquio B disease (MBD; MIM# 253010), are caused by sequence alterations in a single gene, \textit{GLB1}. They result in functional deficits of acid \(\beta\)-galactosidase (\(\beta\)-Gal; EC 3.1.2.23; MIM# 611458), an enzyme cleaving terminal \(\beta\)-linked galactose residues from complex carbohydrates in the lysosomal compartment.

GM1 is a clinically heterogeneous, neurosomatic disease. In its infantile form (type I, OMIM #230500), early developmental arrest is observed followed by progressive neurologic deterioration and spasticity (1). Macular cherry-red spots, facial
dysmorphism, hepatosplenomegaly and generalized dysostosis are usually present. Juvenile (type II, OMIM #230600) and chronic phenotypes (type III, OMIM #230650) display a progressive neurologic disease in childhood or early adulthood with minor or absent dysmorphic changes (1). On the contrary, typical MBD patients completely retain their neurological functions, but develop severe skeletal dysplasia, not distinguishable from attenuated manifestations of mucopolysaccharidosis IV. Impaired degradation of glycopeptides and glycosaminoglycans including keratan sulfate in connective tissues and visceral organs is supposed to cause growth retardation, corneal clouding, and valve thickening in the heart as well as increased urinary excretion of keratan sulfate. Although their neurological functions, but develop severe skeletal dysplasia, not distinguishable from attenuated manifestations of mucopolysaccharidosis IV. Impaired degradation of glycopeptides and glycosaminoglycans including keratan sulfate in connective tissues and visceral organs is supposed to cause growth retardation, corneal clouding, and valve thickening in the heart as well as increased urinary excretion of keratan sulfate. Although these disease phenotypes were first described more than 30 years ago, their pathogenesis is still not fully resolved, most likely due to rather complex molecular mechanisms involved.

The GLB1 gene, located on chromosome 3p21.33, is composed of 16 exons (2,3). Upon expression, alternative splicing results in two different transcripts, a 2.5-kb RNA coding for enzymatically active acid ß-Gal and a minor 2.0-kb RNA encoding the elastin binding protein (EBP) (4), located in the cellular membrane and involved in the assembly of tropoelastin into growing elastin fibers (5,6). It lacks exons 3, 4 and 6 and contains a unique stretch of 32 amino acids due to a different reading frame in exon 5. On the level of gene products, they can include effects on both ß-Gal and EBP (amino acids 1–82 and 245–678) or on ß-Gal alone (amino acids 83–153 and 185–245).

Both GLB1 transcripts have been shown to form multiprotein complexes with neuraminidase, protective protein/cathepsin A (PPCA), and N-acetyl-galactosamine-6-sulfate sulfatase either on the cell surface or in the lysosome, respectively (7,8). Mutations affecting residues on the surface of the ß-Gal molecule are therefore supposed to alter the interaction of ß-Gal or EBP with the other components of the multiprotein complex. Several alleles were shown to influence the multi-enzyme assembly and aggregation (9–11).

ß-Gal cleaves terminal ß-linked galactose residues from structurally diverse natural substrates, such as GM1 ganglioside, N- and O-linked oligosaccharides and keratan sulfate in the lysosomal compartment. Mutations affecting the catalytic site of the enzyme may differentially modify the degradation of natural substrates.

The tertiary structures of ß-Gal or EBP have, however, not been resolved. Therefore, single mutations cannot certainly be correlated with the proposed intra- and intermolecular mechanisms in pathogenesis. Recently, a structural model of human ß-Gal was constructed using crystallographic data for B.thetaiotaomicron ß-Gal as a template (12). Based on an amino acid identity with human ß-Gal of 36%, good correlation between structural changes caused by amino acid substitutions in the ß-galactosidase molecule and biochemical and clinical phenotypes was proposed for three mutations found in an infantile (p.G123R), a juvenile (p.R201C) and an adult (p.I51T) GM1 patient. However, a rare allele (p.Y83H) was chosen for MBD, found in a single, heteroallelic MBD patient (genotype p.Y83H/p.R482C). Therefore, this model has to be completed with data for the major common MBD allele, p.W273L (13) before it can be generally applied.

Nevertheless, for few of the over 100 detected mutations of the GLB1 gene, distinct functional deficits of its gene products can be suggested due to their occurrence in homoallelic cases: p.W273L, the common allele in MBD, has repeatedly been found in homoallelic patients and determines the phenotype if present in heteroallelic patients (13,14). It is located in the catalytical domain and supposed to code for an enzyme with increased activity (>10% of normal) against synthetic substrates, strongly reduced affinity toward keratan sulfate and normal degradation of GM1 ganglioside (15). The enzyme precursor undergoes normal processing and transport to the lysosomes (16,17). Some other common alleles (p.I51T, p.T82M, p.R201H, p.R201C) found in homozygous patients with juvenile or adult GM1 also exhibit increased residual activities against synthetic substrates. They were shown to express enzyme precursors with impaired stability, diminished aggregation with other components of the multienzyme complex and rapid subsequent degradation (18,19), whereas in severe infantile patients fibroblasts, ß-Gal activity against synthetic substrates is very low (<1%) and a complete lack of ß-Gal protein may be present (15). Moreover, some GM1 mutations were shown to protect patients from developing the most severe form of GM1 (19–21).

Our group recently published further clinical, biochemical, immunohistochemical and molecular data on GM1 and MBD patients and an expression analysis of novel pathogenic alleles in cultured cells (22). We now report on the detection of novel pathogenic alleles in the GLB1 gene and estimate the phenotype specificity of alleles in 10 heteroallelic patients, based on empirical data from
Phenotype determining alleles in GM1 gangliosidosis patients

Materials and methods

Patients

After diagnosis of GM1 gangliosidosis in 16 patients, genetic analysis of the GLB1 gene was performed. Their clinical data were collected by several coauthors (MB; AR, AV, BJP, HM, BP) and by the colleagues mentioned in the acknowledgements section using the same common questionnaire (Table 1).

DNA isolation, amplification, and restriction analysis

DNA was isolated from skin fibroblasts or blood using the QIAamp Blood Mini Kit (Qiagen, Hilden, Germany). The genomic DNA was subjected to PCR for amplification of the 16 exons and the flanking regions of the GLB1 gene. PCR conditions have recently been described (22). cDNA reference sequence is GenBank NM_000404.2. The identified mutations were confirmed by restriction analysis (Table 2) with enzymes purchased from New England Biolabs (Frankfurt, Germany) and Roche (Vienna, Austria). For the exclusion of polymorphisms, at least 100 control alleles were analyzed for each novel mutation. The novel intronic mutation c.734-8A>G could not be found in 230 normal DNA samples.

Site-directed mutagenesis

Introduction of mutations into the wildtype DNA was performed using the QuikChange II Site Directed Mutagenesis Kit (Stratagene, La Jolla, California) following the manufacturer’s manual. Template was the wildtype ß-Gal cDNA in the modified pcDX vector, kindly provided by A. Morrone, Department of Paediatrics, University of Florence, Children’s Hospital ‘A.Meyer’, Italy, and A. d’Azzo, Department of Genetics and Tumor Cell Biology, St. Jude Children’s Research Hospital, Memphis, TN. Mutagenesis was confirmed by restriction analysis (Table 2). In addition to the expression of the newly identified mutations, three different artificial nonsense mutations in exon 16 (p.W582X, p.E620X, p.K659X) were constructed to assess residual enzyme activity of truncated ß-Gal mutants.

Expression analysis

Confluent COS-1 cells were transfected in 12-well trays with 1.2 µg wildtype or mutant DNA using

<table>
<thead>
<tr>
<th>Patient</th>
<th>Genotype</th>
<th>Clinical phenotype</th>
<th>Age at diagnosis</th>
<th>Onset</th>
<th>Ethnic origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>c.245+1G&gt;A, c.245+1G&gt;A</td>
<td>Infantile GM1</td>
<td>9 months</td>
<td>6 months</td>
<td>Turkish</td>
</tr>
<tr>
<td>3</td>
<td>p.I181K/p.I181K</td>
<td>Infantile GM1</td>
<td>1 year</td>
<td>6 months</td>
<td>Turkish</td>
</tr>
<tr>
<td>4</td>
<td>p.W161X/p.R208C</td>
<td>Infantile GM1</td>
<td>1 year</td>
<td>11 months</td>
<td>German</td>
</tr>
<tr>
<td>5</td>
<td>p.C230R/p.C230R</td>
<td>Infantile GM1</td>
<td>1 year</td>
<td>0 months</td>
<td>Moroccan</td>
</tr>
<tr>
<td>6</td>
<td>p.W273X/p.D448V</td>
<td>Infantile GM1</td>
<td>1 year</td>
<td>1 year</td>
<td>Turkish</td>
</tr>
<tr>
<td>7</td>
<td>p.C127Y/p.C127Y</td>
<td>Juvenile GM1</td>
<td>2 years</td>
<td>1 year</td>
<td>Pakistani</td>
</tr>
<tr>
<td>8</td>
<td>p.F107L/p.D448GfsX8</td>
<td>Juvenile GM1</td>
<td>2 years</td>
<td>2 years</td>
<td>Greek</td>
</tr>
<tr>
<td>9</td>
<td>p.I55FfsX16/p.R201H</td>
<td>Juvenile GM1</td>
<td>2 years</td>
<td>4 years</td>
<td>British</td>
</tr>
<tr>
<td>10</td>
<td>p.A301V/p.L389P</td>
<td>Juvenile GM1</td>
<td>2 years</td>
<td>6 months</td>
<td>German</td>
</tr>
<tr>
<td>11</td>
<td>p.R49H/p.G526GfsX5</td>
<td>Adult GM1</td>
<td>2 years</td>
<td>1 year</td>
<td>Honduran</td>
</tr>
<tr>
<td>12</td>
<td>p.T82M/p.H112P</td>
<td>Adult GM1</td>
<td>2 years</td>
<td>6 years</td>
<td>British</td>
</tr>
<tr>
<td>13</td>
<td>p.W65X/p.L155R</td>
<td>Adult GM1</td>
<td>2 years</td>
<td>4 years</td>
<td>German</td>
</tr>
<tr>
<td>14</td>
<td>p.K73E/p.R148C</td>
<td>Adult GM1</td>
<td>4 years</td>
<td>4 years</td>
<td>Moroccan</td>
</tr>
<tr>
<td>15</td>
<td>p.L155R/p.R299VfsX5</td>
<td>Adult GM1</td>
<td>4 years</td>
<td>4 years</td>
<td>Turkish</td>
</tr>
<tr>
<td>16</td>
<td>p.F357L</td>
<td>Adult GM1</td>
<td>4 years</td>
<td>16 years</td>
<td>Pakistani</td>
</tr>
</tbody>
</table>

CNS, central nervous system involvement; CRS, cherry-red spots; CI, cardiac involvement; HS, hepatosplenomegaly; SA, skeletal affection; n.a., not available.
4 μl Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany), according to the manual. Negative control was the pcDX vector without insert. After 20 h of incubation, the cells were harvested by scraping and resuspended in 0.9% NaCl containing 0.01% Triton X-100. The cell suspension was sonicated for lysis and β-Gal activity, and protein content was determined in the supernatant. Fibroblasts as well as COS-1 cells were maintained in Minimal Essential Medium (MEM) with Earle’s Salts (PAA, Pasching, Austria) containing 10% fetal bovine serum, 400 μM L-glutamine, and 50 μg/ml gentamicine at 37°C and 5% CO₂.

β-Gal and β-hexosaminidase activity were measured using the synthetic substrates 4-methylumbelliferyl-β-D-galactopyranoside and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (Sigma−Aldrich, Taufkirchen, Germany), respectively. Enzymatic hydrolase activity was measured with a luminescence spectrophotometer (Perkin Elmer, Wiesbaden, Germany) according to standard protocols. Determination of total protein content of the harvested cells was performed as described (30).

Western blot analysis

For Western blot analysis of the mutant β-Gal proteins, 20 μg of total protein of COS-1 cell homogenate were separated with 10% SDS-polyacrylamide gel electrophoresis and subsequently transferred to Invitrolon PVDF membranes (Invitrogen, Karlsruhe, Germany). Hybridization with α85 antihuman β-galactosidase antibody directed against the precursor and the mature form of β-Gal (31), kindly provided by A. d’Azzo, Memphis, TN, and with a secondary phosphatase-conjugated goat anti-rabbit IgG (whole molecule) antibody (Sigma–Aldrich, Taufkirchen, Germany) was performed. The alkaline phosphatase color reaction (AP Conjugate Substrate kit; Bio-Rad, Hercules, California) was applied to visualize the β-Gal corresponding protein bands. BenchMark prestained protein ladder was used to estimate the protein size (Invitrogen, Karlsruhe, Germany). Fibroblast cells Western blotting was performed using the same procedure, but 30 μg of total protein of cell homogenate per gel lane were loaded. For PPCA detection, a polyclonal PPCA antibody (Abcam, Cambridge, UK) was used. Access to fibroblast cultures was limited to patients 3, 4, 5, 8.
Phenotype determining alleles in GM1 gangliosidosis patients

10 and 11. Therefore, Western blotting could only be performed in homogenates of these cell lines.

Immunostaining

FITC-staining of the β-Gal in healthy fibroblast cells and six GM1 gangliosidosis cell lines (patients 3, 4, 5, 8, 10 and 14) was performed using the α85 antihuman β-galactosidase antibody and a FITC-conjugated secondary antibody. For detailed experimental conditions refer to (22). The images were acquired using an Olympus IX51 (Olympus, Hamburg, Germany) inverted research microscope and a 100× oil immersion objective for appropriate magnification.

Results

Sequence alterations in the GLB1 gene

Sixteen GM1 gangliosidosis patients of European, Central American, African, and Middle Eastern origin were studied (Table 1). We found 12 compound heterozygous and four homozygous patients; patients 1 and 5 are of consanguineous parents. Out of 28 detected sequence alterations in the genomic DNA of these patients 18 were missense mutations, 3 nonsense mutations, 2 intronic mutations, 1 duplication, 3 deletions, and 1 insertion (Table 2). They are located in several exons among the entire GLB1 gene, with a cluster in exon 2 of 15.6%. As in the duplication, the deletions and the insertion, only one nucleotide was found to be changed; they all result in a frameshift and consequently in a premature stop codon 5 to 23 codons downstream of the mutation site. Thus, they are all likely to result in truncated, enzymatically inactive products. Upon recognition of the premature stop codon, the nonsense-mediated mRNA decay will also play an important role in the removal of the incomplete RNA prior to translation of the truncated protein. Twenty-five percent of all detected genetic lesions occur in codons presenting the potential CpG DNA methylation site (all seven missense mutation altering arginine residues and p.A301V) with increased probability for mutational events (32). Nine missense mutations, three nonsense mutations, one intronic mutation, the duplication, and the three deletions have not been published so far (Table 2).

Expression studies

Overexpressed wildtype β-Gal in COS-1 cells resulted in enzymatic activity of 556.6 ± 123.4 nmol/h/mg protein, whereas the basal activity of COS-1 cells, transfected with pcDX vector devoid of insert, was 71.9 ± 14.9 nmol/h/mg protein. Four mutations, c.335A>C (p.H112P), c.380G>A (p.C127Y), c.542T>A (p.I181K), and c.1343A>T (p.D448V), did not display any β-Gal activity upon expression in COS-1 cells (Table 3). The other mutations expressed, c.146G>A (p.R49H), c.217A>G (p.K73E), c.319T>C (p.F107L), c.688T>C (p.C230R), c.902C>T (p.A301V), and c.1166T>C (p.L389P), showed enzyme activities equal to or below 6.1% residual β-Gal wildtype activity (Table 3). Besides p.C230R, all the other mutations with detectable enzyme activity were

Table 3. β-Galactosidase activity in transiently transfected COS-1 cells

<table>
<thead>
<tr>
<th>β-Galactosidase activity (nmol/h/mg protein)</th>
<th>Percentage of wildtype activity</th>
<th>β-Hexosaminidase activity (nmol/h/mg protein)</th>
<th>Number of replicas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>556.6 ± 123.4</td>
<td>100.0</td>
<td>637.8 ± 90.7</td>
</tr>
<tr>
<td>Basal</td>
<td>71.9 ± 14.9</td>
<td>0</td>
<td>605.1 ± 64.2</td>
</tr>
<tr>
<td>p.R49H</td>
<td>101.4 ± 19.9</td>
<td>6.1</td>
<td>623.5 ± 102.0</td>
</tr>
<tr>
<td>p.K73E</td>
<td>88.2 ± 21.1</td>
<td>3.4</td>
<td>598.4 ± 71.3</td>
</tr>
<tr>
<td>p.F107L</td>
<td>77.8 ± 14.9</td>
<td>1.2</td>
<td>549.4 ± 126.5</td>
</tr>
<tr>
<td>p.H112P</td>
<td>67.9 ± 14.4</td>
<td>0</td>
<td>594.2 ± 74.0</td>
</tr>
<tr>
<td>p.C127Y</td>
<td>63.4 ± 8.7</td>
<td>0</td>
<td>546.7 ± 125.5</td>
</tr>
<tr>
<td>p.I181K</td>
<td>67.4 ± 15.4</td>
<td>0</td>
<td>647.3 ± 63.7</td>
</tr>
<tr>
<td>p.C230R</td>
<td>89.7 ± 17.7</td>
<td>3.7</td>
<td>606.0 ± 120.5</td>
</tr>
<tr>
<td>p.A301V</td>
<td>79.2 ± 19.5</td>
<td>1.5</td>
<td>680.9 ± 102.3</td>
</tr>
<tr>
<td>p.L389P</td>
<td>74.2 ± 17.8</td>
<td>0.5</td>
<td>624.4 ± 162.2</td>
</tr>
<tr>
<td>p.D448V</td>
<td>59.4 ± 10.5</td>
<td>0</td>
<td>572.9 ± 110.3</td>
</tr>
<tr>
<td>p.W582X</td>
<td>62.8 ± 21.6</td>
<td>0</td>
<td>584.45 ± 135.3</td>
</tr>
<tr>
<td>p.E620X</td>
<td>68.0 ± 27.1</td>
<td>0</td>
<td>487.91 ± 124.6</td>
</tr>
<tr>
<td>p.K659X</td>
<td>567.2 ± 89.1</td>
<td>102.2</td>
<td>549.98 ± 155.1</td>
</tr>
</tbody>
</table>
identified in juvenile and adult patients. The significant reduction of enzyme activity in the assayed mutations is in consistency with the pathogenic character of the sequence alterations. Among the expressed artificial nonsense mutations in exon 16, p.W582X and p.E620X did not show any residual enzyme activity, whereas p.K659X displayed the wildtype activity (Table 3).

Western blot analysis

From the detection of β-Gal precursor proteins upon Western blotting of the overexpressing COS-1 cell homogenates, we conclude that stable β-Gal precursors were formed (Fig. 1). Western blotting of fibroblast cell homogenates of affected patients show a faint 64 kDa mature β-Gal band in the patients compared to the control cells (Fig. 2). The 85 kDa precursor band is present in all patient homogenates comparable to the control cell line. Several faint degradation bands are visible in all cell lines tested. Infantile patient 3 (p.I181K/p.I181K) shows a considerable strong degradation band at approximately 40 kDa. The PPCA antibody recognized the precursor band of 54 kDa and the catalytically active 32 kDa and 20 kDa chains. Although the precursor protein is merely present in the wildtype control, the smaller proteins are detected in all patients comparable with the control.

Immunostaining of fibroblasts

In accordance to the presence of β-Gal precursor in Western blots from overexpressing COS-1 cells, β-Gal protein was detected in all six GM1 gangliosidosis fibroblast lines as well as in the control cell line (Fig. 3). The enzyme was distributed in punctate organelles over the entire cell body and there was no difference in fluorescence distribution between healthy controls and the GM1 cell lines. As recently shown with proven transport-deficient mutants, one has to conclude that the mutated proteins were transported into lysosome-related membrane systems (22).

Discussion

In this paper, we describe 18 missense mutations, 3 nonsense mutations, 2 intronic mutations, 3 small deletions, a small insertion, and a duplication detected in the genomic DNA of 16 GM1 gangliosidosis patients (Table 2). Nine of the missense mutations, the three nonsense mutations, the deletions, one of the intronic mutations, and the duplication have not been published so far. Over 100 mutations of the GLB1 gene have to date been shown to cause a broad spectrum of clinical manifestations, ranging from cases with severe early infantile neuronal dysfunction to attenuated skeletal dysplasia without any affection of the central nervous system.
GLB1 mutations may (1) inhibit the catalytic reaction, impair correct protein folding and/or result in the formation of unstable proteins. If located at the active site, they can result in the loss of a catalytically essential amino acid or in a sterical hindrance of substrate access to the catalytic site (2). If affecting the β-Gal surface and thus the multienzyme complex aggregation, enzymatic activity or protein stability may be impaired. (3) If a domain common to β-Gal and EBP is affected, the resulting phenotype may reflect inhibited lysosomal degradation and impaired assembly of extracellular matrix and/or processes related to signal transduction (33-35). (4) Due to the influence of modifier genes, even identical genotypes may not always result in the same phenotypes among different patients (36).

Despite this complex situation, the available results on clinical genetics of the GLB1 gene provide increasing empirical evidence on correlations between single alleles and distinct phenotypes, either due to homoallelic occurrence in single patients or due to the presence of counteralleles resulting in truncated dysfunctional proteins. As summarized in Table 4, the results of this paper correlate well with previous data and may provide a basis for further molecular investigations.

Known specific alleles

Few mutations in the GLB1 gene could so far be clearly related to a specific GM1 or MBD phenotype. Three codons were found to be crucial for MBD: (i) Tyr83 (p.Y83C/p.D441N (38); p.Y83H/p.R482C (39)); (ii) Tyr500 (p.N484K/p.T500A (40); p.L173P/p.T500A (38); p.Q408P/p.T500A (22)), and (iii) Trp273 (p.W273L/p.W273L (14); p.W273R/p.H281Y (41)).

Contrary to the recent in silico-model (12), previous evidence suggests that codon p.W273 is involved in the catalytic function and may stabilize the galactose residue in the catalytic pocket during substrate cleavage (14,15). The common allele p.W273L is supposed to result in a stable protein with altered catalytic function, providing normal degradation to GM1 ganglioside but impaired cleavage of keratan sulfate (16). In one single MBD patient (p.Y444C/p.G494S (38), the phenotype-specific allele could so far not be determined, as no other reports on these alleles are in existence. Other alleles, affecting Arg201 (p.R201H) and Gly438 (p.G438E) have been detected in homozygous patients with either juvenile/adult GM1 or MBD (23,28,34,40,42) and therefore cannot be clearly assigned to a phenotype.

Among others (p.I51T (19); p.L155R (26); p.T420K (42)), p.T82M has repeatedly been shown to result in adult GM1, independent of the counterallele. So far, five homoallelic p.T82M patients were detected. They all presented with a delay of speech development and clumsiness between 2 and 4 years of age, subsequent development of spondyloepiphysed dysplasia and decreased β-Gal activity in fibroblasts or leukocytes between 2.6% and 3.4% of controls (21,22). These five patients appear to be surprisingly uniform in onset and outcome, thus confirming a phenotype determining effect of p.T82M.

Homoallelic patients

GM1 patients 1, 3, 5, and 7 were homoallelic with their mutations directly related to infantile (c.245+1G>A, p.I181K, and p.C230R) and juvenile GM1 (p.C127Y), respectively. Homozygous patient 5 (p.C230R) appears to be of particular notice. Lysosomal storage was suspected at birth due to storage cells in the placenta rather than due to typical GM1 symptoms and final diagnosis achieved at 2 months of age. His early and severe neurological symptoms (severe spasticity, cherry-red spots) and poor cognitive outcome until to his current age of 2 were consistent with a classification as infantile GM1 (1) despite a proven...
Hofer et al.

lack of cardiac dysfunction, the absence of hepatosplenomegaly and only minor symptoms of bone disease. Some of the visceral symptoms may have been ameliorated by stem cell transplantation at 4 months of age. However, β-Gal activity in fibroblasts (3.5% of wildtype) and the activity of mutant precursors after overexpression in COS-1 cells (3.7% of wildtype) were similar to GM1 type 2 and 3 and may account for the relatively mild organ affection and minor dysmorphism.

As p.C230R is located in exon 6, which is skipped in the alternatively spliced GLB1 gene product, EBP, it can be supposed to be fully functional. In accordance with previous reports, this would imply normal elastogenesis and consequently normal cardiac function (33). Similar to other atypical phenotypes of the GLB1 gene, knowledge on the consequences of the C230R allele on protein function, including the degradation of natural substrates is required for an explanation of the relatively high residual activity of mutant β-Gal in the severely affected patient 5.

Heteroallelic patients

As little was known on the potential residual activity of truncated β-Gal mutants, three artificial nonsense mutations (p.W582X, p.E620X and p.K659X) were introduced in exon 16 (Table 4). In accordance with previous results on the importance of the C-terminus for β-Gal function (31), p.W582X and p.E620X did not result in any residual activity. Merely, p.K659X, located 19 amino acids prior to the natural stop codon, had normal catalytic activity. In exon 16, no naturally occurring termination mutation has so far been identified and p.W576X was the mutation located closest to the natural stop codon (26). Thus, all detected nonsense mutations resulting in shorter truncated proteins than the introduced termination codons have to be devoid of β-Gal activity. In heteroallelic patients with nonsense mutations, the counterallele has thus to be determinant for the phenotype, as illustrated in the infantile GM1 patients 4 and 6 bearing the missense mutations p.R208C and p.D448V, respectively, besides nonsense mutations on the other allele. Expression products of p.D448V in COS-1 cells were completely devoid of enzymatic activity. Furthermore, patient 6 (p.W273X/p.D448V) would have no functional β-Gal enzyme at all, while for patient 4 (p.W161X/p.R208C), any potential β-Gal activity would be attributable to p.R208C, similar to a homozygous patient. This is consistent with results from two homozygous patients with identical onset of disease during the second half-year of life (27).

As listed in Table 4, several other mutations can be related to juvenile and adult GM1 by similar considerations: p.R201H is well-known to protect patients from severe GM1 (26,28,34,38) and consistently patient 9, genotype p.I55FfsX16/p.R201H, was manifested as juvenile GM1. In analogy, p.F107L, displaying 1.2% of normal β-Gal activity upon overexpression, has to be determinant for juvenile patient 8, as the counter allele p.D448GfsX8 results in a truncated β-Gal protein. p.L155R, previously found in a homozygous patient classified as GM1 type 3 with gait and speech disturbances at the age of 6 (26), has to be dominant in the patients 14 (p.W65X/p.L155R) and 15 (p.L155R/p.R299VfsX5).

Mutation p.R442Q has so far been found in patient 16 and in another adult patient (28), implicating a significance for adult GM1. Despite single reports on neighboring missense mutations resulting in the severe phenotype, a conspicuous clustering of mutations associated with mild GM1 or MBD in exon 13, with minor impact on β-Gal function can be proposed (23,26,38,40).


Juvenile patient 10 carries the mutations p.A301V and p.L389P, resulting in 1.5% and 0.5% of wildtype activity upon COS-cells expression, respectively. Both mutations have not been detected so far and no conclusions on the phenotype-determinant allele can be drawn.

Taken together, 17 novel sequence alterations in the GLB1 gene could be described. Upon comparison of our results with previous data, phenotype specificities in heteroallelic genotypes were proposed for nine GLB1 mutations. Future observations, biochemical experiments and data on molecular structure of the β-Gal protein have to confirm their predictive value for GM1 gangliosidosis patients.

Acknowledgements

This work was supported by the Austrian Ministry of Science and Research (single project GZ 200.156/2-V/1a/2006 entitled ‘Investigations for the Early Diagnosis and Prognosis of the Phenotype...
Hofer et al.
in Lysosomal Storage Diseases’) and the Austrian Research Society for Mucopolysaccharidosis and Related Diseases. The authors are indebted to A. Morrone, University of Florence, Italy, and A. d’Azzo, St. Jude Children’s Research Hospital in Memphis, TN, for helpful discussions and provision of the GLBI pcDX-X cDNA and the αs8 antihuman-β-galactosidase antibody. Careful reports on clinical data were provided by D. Mansour, Department of Contraception and Sexual Health, Graingerville Clinic, Newcastle General Hospital, UK, K. Tylee, Willink Biochemical Genetics Unit, Royal Manchester Children’s Hospital, Manchester, UK and F.S. Ezgu, Gazi University Hospital Ankara, Turkey. We gratefully acknowledge the technical assistance of Bettina Pabst and Tania Skachkova and the patients’ cooperation for providing fibroblasts cultures.

Conflicts of interest
All authors state that there are no actual or potential conflicts of interest in the present study, including any financial, personal or other relationships with other people or organizations.

References
Phenotype determining alleles in GM1 gangliosidosis patients