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ARTICLE

Functional analysis of 11 novel *GBA* alleles

Erika Malini[1], Serena Grossi[2], Marta Deganuto[1], Camillo Rosano[3], Rossella Parini[4], Silvia Dominisini[1], Roberta Cariati[1], Stefania Zampieri[1], Bruno Bembi[1], Mirella Filocamo[2] and Andrea Dardis*[,1]

Gaucher disease is the most frequent lysosomal storage disorder due to the deficiency of the acid β-glucosidase, encoded by the *GBA* gene. In this study, we report the structural and functional characterization of 11 novel *GBA* alleles. Seven single missense alleles, P159S, N188I, E235K, P245T, W312S, S366R and W381C, and two alleles carrying *in cis* mutations, (N188S; G265R) and (E326K; D380N), were studied for enzyme activity in transiently transfected cells. All mutants were inactive except the P159S, which retained 15% of wild-type activity. To further characterize the alleles carrying two *in cis* mutations, we expressed constructs bearing singly each mutation. The presence of G265R or D380N mutations completely abolished enzyme activity, while N188S and E326K mutants retained 25 and 54% of wild-type activity, respectively. Two mutations, affecting the acceptor splice site of introns 5 (c.589-1G>A) and 9 (c.1389-1G>A), led to the synthesis of aberrant mRNA. Unpredictably, family studies showed that two alleles resulted from germline or '*de novo*' mutations. These results strengthen the importance of performing a complete and accurate molecular analysis of the *GBA* gene in order to avoid misleading conclusions and provide a comprehensive functional analysis of new *GBA* mutations.
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INTRODUCTION

Gaucher disease (GD) is the most frequent lysosomal storage disorder due to the deficiency of the lysosomal hydrolase, acid β-glucosidase (GBA; EC 3.2.1.45). The enzyme is present in the lysosomes of all nucleated cells and cleaves the β-glucosidic linkage of glucosylceramide (GlcCer) yielding glucose and ceramide. The deficiency of GBA leads to the progressive lysosomal accumulation of GlcCer and other glycosphingolipids resulting in multiorgan dysfunction.[1]

The disease has been classically classified into three major clinical variants based on the presence and progression of central nervous system involvement. Type 1 GD (MIM No. 230800), the most common phenotype, is characterized by enlargement and dysfunction of the liver and spleen, displacement of normal bone marrow by storage cells and bone damage leading to infarctions and fractures. Although type 1 GD is considered a non-neuronopathic form, there is increasing evidence of neurological involvement (ie Parkinson syndrome, seizures, oligophrenia, perceptive deafness). Type 2 GD (MIM No. 230900) is a rare phenotype associated with an acute neurodegenerative course and death at a very early age. These patients commonly present with evidence of brainstem dysfunction followed by progressive deterioration, dysphagia, pyramidal signs, failure to thrive and cachexia during the first month of life. Type 3, the chronic neuronopathic GD (MIM No. 231000), comprises an extremely heterogeneous group of patients who present with either mild or severe systemic disease associated with some form of neurological involvement and with an onset of symptoms that might range from childhood to early adulthood.[1]

The human GBA is encoded by the *GBA* gene (*GBA*; MIM No. 606463; GenBank accession no. J03059.1), located on chromosome 1q21. *GBA* gene is approximately 7.5-kb long and contains 11 exons. A highly homologous 5.5 kb-pseudogene (*GBAP*; MIM No. 606463; GenBank accession no. J03060.1) has been located 16 kb downstream from the active gene.[2] More than 300 mutations in the *GBA* gene have been reported to date, including all kind of defects such as single base changes, splicing alterations, insertions, partial and total deletions and gene-pseudogene rearrangements (www.hgmd.org).[3]

GBA protein is synthesized on polyribosomes as a 55-kDa peptide, which is then translocated into the endoplasmic reticulum (ER), where it is modified by the addition of high mannose oligosaccharides, and transported to the trans-Golgi network, from where it is trafficked to the lysosomes.[4] GBA protein is targeted to the lysosomal compartment through a mannose 6-phosphate-independent receptor, the lysosomal integral membrane protein type 2,[5] a trans-membrane protein mainly found in the lysosomes and late endosomes.[6,7]

In this study, we report the identification and functional characterization of 11 novel *GBA* mutations.

MATERIALS AND METHODS

Patients

Novel *GBA* mutations were found in 10 patients (pts) diagnosed with GD disease based on the demonstration of reduced GBA activity in white blood cells or cultured cell lines (fibroblasts and/or EBV-lymphoblasts). According to clinical parameters, including onset, visceromegaly, bone disease and neurological signs, 7 pts were classified as GD1, 1 pt as GD2 and 2 pts as GD3.[1]

Patients' cell lines

Fibroblast and lymphoblast cells from the two patients carrying splicing mutations were cultured and maintained in RPMI medium (EuroClone,

[1]Regional Coordinator Centre for Rare Diseases, University Hospital Santa Maria della Misericordia, Udine, Italy; [2]U.O.S.D. Centro di Diagnostica Genetica e Biochimica delle Malattie Metaboliche, Istituto G. Gaslini, Genova, Italy; [3]Patologia Molecolare Integrata – A.O.U. IRCSS San Martino – IST, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy and [4]Rare Metabolic Diseases Unit, Pediatric Clinic, San Gerardo Hospital, Monza, Italy
*Correspondence: Dr A Dardis, Regional Coordinator Center for Rare Diseases, University Hospital Santa Maria della Misericordia, Udine Piazzale Santa Maria della Misericordia 15, Udine 33100, Italy. Tel: +39 0432 554472; Fax: +39 0432 554472; E-mail: dardis.andrea@aoud.sanita.fvg.it
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Gibco, Paisley, UK) containing 15% of fetal calf serum (FCS) and penicillin/streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

Ethical aspects

Following ethical guidelines, all cell and nucleic acid samples were obtained for analysis and storage with the patients' (and/or a family member's) written informed consent. The consent was sought using a form approved by the local Ethics Committee.

Molecular analysis

Genomic DNA was extracted from peripheral blood leukocytes, cultured fibroblasts and lymphoblasts using QIAamp DNA blood Mini Kit (Qiagen GmbH, Hilden, Germany) or Nucleon BACC3 kit (Amersham Biosciences, Buckinghamshire, UK).

Total RNA was extracted from cell lines using Rneasy mini Kit (Qiagen). First-strand cDNAs were synthesized by Advantage RT-for-PCR Kit (Clontech, Mountain View, CA, USA) and random hexamer primers.

GBA gene exons and most intronic regions were PCR amplified using primers designed by reference to the genomic sequence (GenBank J03059.1) to selectively amplify the gene and not the homologous pseudogene (GenBank J03060.1) as previously described by Koprivica *et al*.[8]

Reverse transcriptase-PCR (RT-PCR) was performed using sets of primers designed by reference to the *GBA* mRNA sequence (GenBank accession No. NM_000157.3), as previously reported.[9] PCR products were cloned into the TOPO TA Cloning KIT (with pCR2.1-TOPO vector) (Invitrogen, San Diego, CA, USA), according to the manufacturer's instructions. PCR and RT-PCR products were analyzed by automated sequencing (ABI Prism 3500xl genetic analyzer, Applied Biosystems, Foster City, CA, USA). Putative mutations were confirmed by sequencing duplicate PCR products and by the DNA analysis from parents and relatives whenever possible. The issue of whether novel *GBA* sequence alterations detected were causative mutations or neutral polymorphisms was addressed by (i) searching dbSNP http://www.ncbi.nlm.nih.gov/SNP) for their presence, (ii) screening 100 alleles from healthy control subjects for each alteration, and (iii) '*in vitro*' expression studies and structural 3D analysis.

Novel GBA alleles have been submitted to the locus-specific mutation database: http://www.hgvs.org/dblist/glsdb.htm.

Variable number tandem repeat (VNTR) profiles were analyzed by Cell ID System Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Site-directed mutagenesis

The novel point mutations of *GBA* gene were introduced in the wild-type full-length cDNA cloned in pcDNA3 already described[10] by site-directed mutagenesis using the Quikchange Site-directed Mutagenesis Kit (Stratagene, Cedar Creek, TX, USA) according to manufacturer's instructions. The sequence of primers used for mutagenesis are listed in Supplementary Table S1.

Mutation nomenclature

All mutations are described according to the current mutation nomenclature guidelines (http://www.hgvs.org/mutnomen), ascribing the A of the first ATG translation initiation codon as nucleotide +1 (GenBank NM_000157.3).[11,12] Traditional amino-acid residue numbering, which excludes the first 39 amino acids of the leader sequence, has nevertheless also been provided in parentheses and designed without the prefix 'p'.

Transient transfection

Human HEK293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco, Paisley, UK) at 37 °C in a humidified atmosphere enriched with 5% (v/v) CO_2. Cells were transfected with the 4 μg of wild-type and mutant constructs using Lipofectammine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 72 h, cells were harvested and the cellular extracts were analyzed for GBA activity.

GBA activity

Total amount of protein in cell lysates was determined by Lowry's method. Ten micrograms of the protein were assayed for GBA activity in 150 μl of buffer citrato 0.1 M –phosphate 0.2 M pH 5.2 containing 0.15% taurocholate (w/v, Calbiochem, La Jolla, CA, USA) in the presence of 7.5 mM 4-MUG for 2 h at 37 °C. The reaction was stopped by the addition of 2 ml of stop solution (buffer carbonato 0.5 M, pH 10.7). The amount of 4-methyl-umbeliferone (4-MU) was quantified using a Gemini XPS Spectrometer (Molecular Devices, Sunnyvale, CA, USA) (excitation length: 340 nm; emission: 495 nm).[13] The Hek293 endogenous GBA activity was measured in cells transfected with empty pcDNA3 vector and subtracted from activity obtained in cells transfected with the WT and mutant constructs. Results were expressed as the percentages of wild-type enzyme activity.

Structural 3D analysis

Visual inspection of GBA three-dimensional structure[14] (PDB code 1OGS) and amino-acid substitutions were carried over using the program Coot;[15] energy minimization using the program DISCOVER3 from the InsightII program suite (Accelrys, Inc., San Diego, CA, USA). Figures were drawn using the program Chimera.[16]

The non-canonical disulfide bridges were predicted using the program http://clavius.bc.edu/~clotelab/DiANNA/.

RESULTS AND DISCUSSION

The 11 novel *GBA* alleles of the present study have been identified during the routine molecular testing of GD patients performed at two Italian laboratory reference centers (Udine and Genoa). Among them, 9 alleles carried 11 missense mutations that occurred as (i) single point mutations c.592C>T [p.P198S (P159S)], c.680A>T [p.N227I (N188I)], c.820G>A [p.E274K (E235K)], c.850C>A [p.P284T (P245T)], c.1052G>C [p.W351S (W312S)], c.1215C>A [p.S405R (S366R)], c.1260G>C [p.W420C (W381C)] or (ii) *in cis* on the same allele: c.680A>G;c.910G>C [p.N227S;p.G304R (N188S;G265R)], c.1093G>A;c.1255G>A [p.E365K;p.D419N (E326K;D380N)]. Finally, two were splice site alterations c.589-1G>A, [p.I197Vfs*7 (I158Vfs*7)] and c.1389-1G>A [p.L464Sfs*24 (L425Sfs*24)] (Table 1).

Table 1 Characteristics of the *GBA* gene mutations identified in Gaucher disease patients

		Predicted effect on protein	
Genomic location	*Site of nucleotide substitution*[a]	*Amino-acid change*[b]	*Traditional numbering*
Ex 6	c.592C>T	p.P198S	P159S
Ex 6	c.680A>T	p.N227I	N188I
Ex 7	c.820G>A	p.E274K	E235K
Ex 7	c.850C>A	p.P284T	P245T
Ex 8	c.1052G>C	p.W351S	W312S
Ex 8	c.1215C>A	p.S405R	S366R
Ex 9	c.1260G>C	p.W420C	W381C
Ex 6 and Ex 7	c.680A>G;c.910G>C	p.N227S;p.G304R	N188S;G265R
Ex 8 and Ex 9	c.1093G>A;c.1255G>A	p.E365K;pD419N	E326K;D380N
Intr 5	c.589-1G>A	p.I197Vfs*7	I158Vfs*7
Intr 9	c.1389-1G>A	p.L464Sfs*24	L425Sfs*24

Abbreviations: Ex, exon; Intr, intron; *GBA* gene, GenBank accession no. J03059.1.
[a]*GBA* cDNA accession number NM_000157.3.
[b]*GBA* cDNA accession number NP_000148.2.

Functional analysis of novel missense *GBA* alleles

The impact of missense mutations on GBA activity was evaluated by *in vitro* expression of mutant constructs in Hek293 cells and the analysis of the expressed GBA activity. As a negative control, Hek293 cells were transfected with empty pcDNA3 vector. The activity of proteins bearing the common p.N409S (N370S) and p.L483P (L444P) mutations were also analyzed. No changes in the viability of transfected cells were observed.

As shown in Figure 1, GBA proteins carrying the changes p.N227I (N188I), p.E274K (E235K), p.P284T (P245T), p.W351S (W312S), p.S405R (S366R) and p.W420C (W381C) presented a very low or absent GBA activity as compared with the wild-type protein, while the p.P198S (P159S) retained a residual activity of 15% of wild type. Both alleles carrying two *in cis* mutations [p.N227S;p.G304R (N188S;G265R)] and [p.E365K;p.D419N (E326K;D380N)] led to the expression of completely inactive proteins. To further characterize these alleles, we expressed *in vitro* constructs bearing singly each mutation. Although the G265R and D380N mutants expressed no activity, both N188S and E326K mutants retained a residual activity of 25 and 54%, respectively. Our data are in line with previous *in vitro* studies, which described the N188S as a modifier variant[17] and the E326K as a very mild mutation.[18] However, while the N188S has been found as a single mutation in GD patients, the E326K has always appeared in association with another mutation on the same allele. As previously reported, mutants N370S and L444P retained a residual activity of 38 and 13% of WT, respectively.[19]

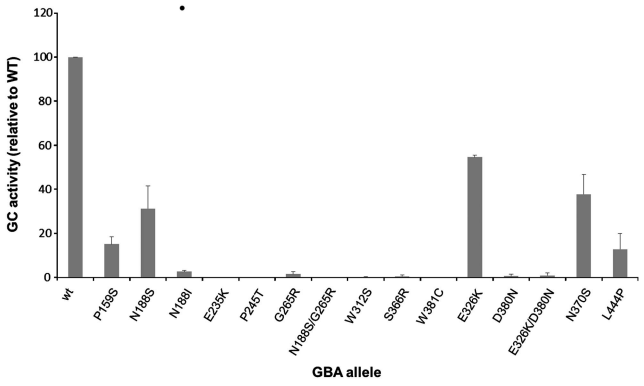

Figure 1 Functional analysis of novel missense GBA alleles. Acid β-glucosidase activity in Hek293 cells transiently transfected with the wild-type and mutant constructs was measured using the fluorogenic substrate 4-MU-D-glucopyranoside. Values are expressed as the percentages of wild-type enzyme activity. The data are shown as mean ± SD of three different experiments, each performed in triplicate.

Structural analysis

To shed further light on the possible consequences of these amino-acid changes at protein level, we performed a structural protein analysis based on the 3D model of the GBA. All mutated residues belong to domain III, which contains the catalytic site[14] (Figure 2). Significantly, the computational analysis revealed that, apart from p.P198S (P159S), p.G304R (G265R) and p.E365K (E326K), all mutations are directly or indirectly involved in substrate recognition (Supplementary Table S2).

Mutations affecting residues located on the protein surface may interfere with the correct coupling of GBA with protein partners [p.P198S (P159S), p.G304R (G265R), p.E365K (E326K)] or indirectly interfere with the catalytic activity of p.N227 (N188): in particular, the '*in vitro*' expression of this latter residue replaced with isoleucine or serine showed that the p.N227I (N188I) mutant was completely inactive, whereas the p.N227S (N188S) mutant retained a quite high residual activity. Indeed, the replacement of a small asparagine with a big and hydrophobic isoleucine (N188I) *versus* a small and polar serine (N188S) is likely to impact upon the correct substrate positioning.

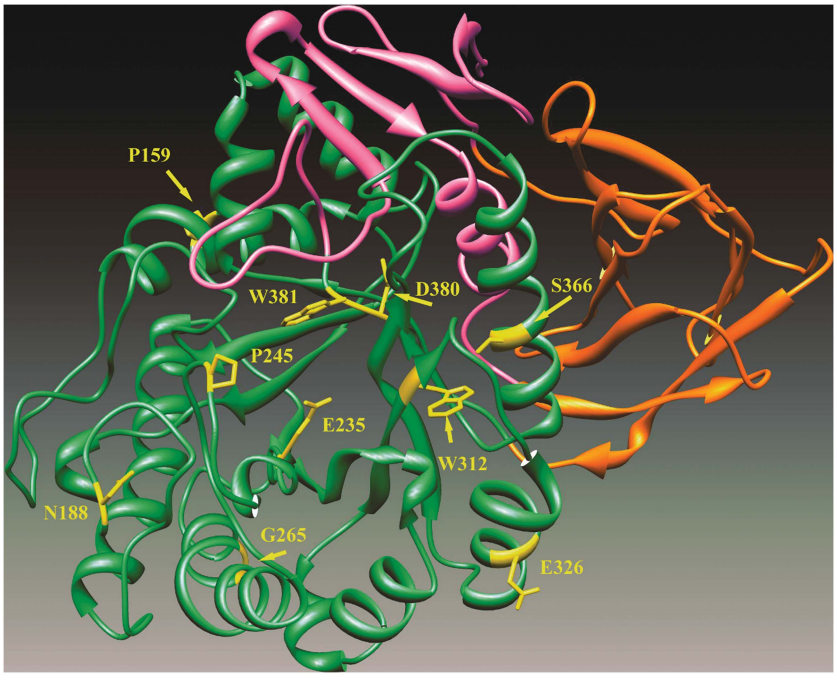

Figure 2 Location of GBA mutations on the X-ray structural model. Catalytic domain is represented in green while the N terminal and C terminal domains are drawn in orange and red, respectively. Positions of the mutations are indicated in yellow.

Functional analysis of splicing mutations

Two intronic mutations affected the invariant acceptor 'G' nucleotide splice site of intron 5 (c.589-1G>A) and intron 9 (c.1389-1G>A). To analyze the possible effect on the mRNA splicing process, we first evaluated the splice site signal strengths using two splicing prediction programs (ME = Maximum Entropy available at:http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq_acc.html[20] and NN = Neural Network available at http://www.fruitfly.org/seq_tools/splice.html.[21] Both programs predicted that the mutated sequence would no longer be recognized as a 3′ splice site or would be recognized with a much lower score than the wild-type sequence (Table 2).

To confirm the impact of these two mutations upon the *GBA* mRNA splicing process *in vivo*, a RT-PCR analysis on mRNAs from the cultured fibroblasts of the respective patients was carried out. Sequence analysis of the RT-PCR products confirmed the exon-6 skipping resulting from c.589-1G>A and showed instead that the intronic c.1389-1G>A led to a slippage of the splice site of four nucleotides in an attempt to restore the invariant acceptor 'AG' nucleotide splice site of intron 9 with the close c.1392G nucleotide of exon 10 (Figure 3). However, this cryptic acceptor splice site was not predicted by either of the *in silico* analysis tools used in this study.

Both intronic mutations, c.589-1G>A and c.1389-1G>A, predicting to introduce premature stop codons resulted in the frameshifts p.I197Vfs*7 and p.L464Sfs*24, respectively.

Phenotype–genotype considerations

As mentioned above, almost all new mutant alleles were predicted to abrogate the GBA activity or cause severe splicing defects (Figure 1 and 3). However, as shown in Table 3, whenever these deleterious alleles were in compound heterozygous patients in association with the common p.N409S (N370S) mutation caused GD type 1; likewise, alleles p.N227I (N188I) and p.E274K (E235K) in association with already known deleterious lesions, p.R170C (R131C) and p.L483P (L444P), respectively, underlay the severe neuronopathic GD type 2.

Much less expected was the GD type 1 phenotype in the patient compound heterozygous for the new [p.W351S (W312S)] mutation, which completely abrogates the GBA activity (Figure 1) in association

Table 2 Predicted effect of *GBA* splicing mutations using bioinformatics tool

	Splicing program	
Mutation	*ME*	*NN*
c.589-1G>A	WT 3′ ss 9.04	WT 3′ ss 0.98
	Mutant 3′SS 0.29	Mutant 3′SS NR
c.1389-1G>A	WT 3′SS 7.66	WT 3′SS 0.38
	Mutant 3′ss 1.09	Mutant 3′ss NR

Abbreviations: ME, Maximum entropy, NN, neural network, NR, not recognized because below detection threshold; WT, wild type.

Table 3 Phenotype–genotype correlation analysis

Genotype[a]		
Allele 1	*Allele 2*	*Phenotype*
[p.P284T (P245T)]	[p.N409S (N370S)]	1
[p.S405R (S366R)]		
[p.W420C (W381C)]		
[p.I197Vfs*7 (I158Vfs*7)]		
[p.L464Sfs*24 (L425Sfs*24)]		
[p.W351S (W312S)]	[p.L483P (L444P)]	1
[p.N227I (N188I)]	[p.R170C (R131C)]	2
[p.E274K (E235K)]	[p.G241R (G202R)]	2
[p.E365K;p.D419N (E326K;D380N)]	[p.P198S (P159S)]	1
[p.N227S;p.G304R (N188S;G265R)]	Unknown	3

[a]Traditional amino-acid residue numbering, which excludes the first 39 amino acids of the leader sequence, has been provided in parentheses and designed without the prefix 'p.'

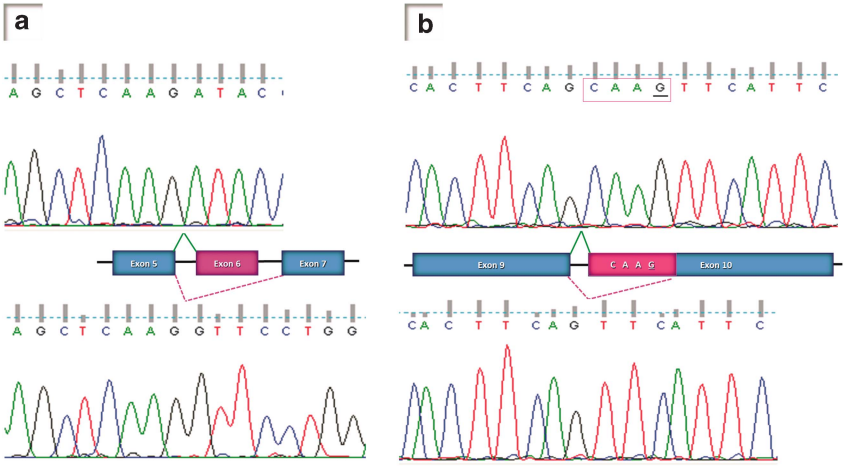

Figure 3 Functional analysis of the two splicing mutations. Results of the reverse transcriptase-PCR analysis on mRNA samples showed that the c.589-1G>A mutation caused the abrogation of intron 5 acceptor 3′ splice site (ss) and the skipping of exon 6 (**a**), whereas the c.1389-1G>A mutation led to the abrogation of intron 9 acceptor 3′ss and activation of alternative 3′ss in exon 10 (4 nucleotides downstream of the canonical 3′ss using the c.1392G nucleotide) (**b**). Normal splicing is depicted as unbroken green lines and abnormal splicing as dotted fucsia lines; blue boxes indicate normally spliced exons and fucsia boxes genomic regions involved in the altered splicing process.

with the [p.L483P (L444P)]. This patient, who was diagnosed as having GD and treated with ERT at the age of 9 years, is indeed a 22-year-old woman with no evidence of clinical or subclinical neurological involvement. In an attempt to exclude the theoretical possibility of additional *in cis* mutations, both the alleles of this patient have been entirely sequenced. In circumstances like these, it is particularly difficult to evaluate how the therapy, on one hand, and other modifying genetic or nongenetic factors, on the other hand, might have contributed to such an unexpected clinical course of the disease.

Splenomegaly, anemia and growth retardation were the presenting symptoms of a 5-year-old GD1 patient compound heterozygous for two new alleles: one carrying two *in cis* mutations [p.E365K;p.D419N (E326K;D380N)] in association with the p.P198S (P159S) allele. The patient, who has been treated with ERT since the age of 6 years, is currently an 11-year-old girl with no evidence of neurological involvement. It is therefore likely that 15% of residual activity retained by the protein bearing the p.P198S (P159S) mutation (Figure 1) might account for the mild phenotype of the patient. The '*in vitro*' expression findings appear also to be in line with the structural protein analysis (Figure 3).

Finally, the other allele carrying two *in cis* mutations [p.N227S;p.G304R (N188S;G265R)] was found in a GD type III patient, of whom the second allele remained undetermined likely owing to some molecular lesions escaping conventional sequencing analysis.[22] However, the limited samples from the patient did not allow us to extend the analysis for revealing lesions such as recombinations, large deletions or deep intronic mutations potentially altering the mRNA processing. Even in the absence of a complete genotype, some important considerations can be made in this patient based on the presented results. Results of the '*in vitro*' expression of each single mutation showed that while the enzymatic activity of p.G304R (G265R) mutant was nearly absent, the p.N227S (N188S) mutant retained a quite high residual activity, as also reported in previous studies.[17] Conversely, the cumulative effect of the double mutant seemed to be highly deleterious as the *in vitro* expressed activity was almost undetectable. Interestingly, this patient developed myoclonic epilepsy, previously reported as associated with the p.N227S (N188S) mutation.[23,24] It is therefore likely that myoclonic epilepsy is always determined by the p.N227S (N188S) both as single mutation and as part of a double-mutant allele.

Even though some phenotype–genotype considerations can be supported by the presented results, it must be acknowledged that the *in vitro* expression model is not entirely suitable to study the effect of GBA mutations on protein processing, one of the main factors that determine GD severity.[25] In fact, even if some GBA mutations lead to the synthesis of partially active proteins, they are retained in the ER, due to their inability to fold correctly, undergo ER-associated proteasomal degradation and never reach the lyosomes.[26] In our experimental set, the overexpression of GBA proteins results in a saturation of the systems involved in their trafficking and degradation, rendering the model not suitable to evaluate protein fate.

Finally, it is important to keep in mind that besides GBA mutations, genetic variations in proteins involved in GBA processing may also have a role in the phenotypic expression of GD.

Family studies

The mutation analysis was extended wherever possible to all the family members. Quite unpredictably, we came across two germline or '*de novo*' mutations, the p.D419N (D380N), found in association with another point mutation in the same allele [p.E365K;p.D419N (E326K;D380N)] and the [p.G241R (G202R)]. As both mutations occurred on the paternally derived allele, various samples were used to exclude the theoretical possibility of a sample exchange as well as VNTR profiles were compared in the two families to exclude mispaternity. So far, '*de novo*' or germline mutations appears to be extremely rare in Gaucher disease.[27,28] However, since such events have significant implications for genetic counselling, we emphasize the importance of the families' studies. Gonadal mosaicism could not be formally excluded in the respective patient's fathers who were found to be negative with respect to a specific mutation. Therefore, in clinical practice, prenatal testing is recommended for those couples still potentially at risk.

These results strengthen the importance of performing a complete and accurate molecular analysis of the *GBA* gene in order to avoid misleading conclusions and to provide an appropriate genetic counselling. In addition, they provide a comprehensive functional analysis of new GBA mutations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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