Spectrum of *GBA* Mutations in Patients with Gaucher Disease from Slovakia: Identification of Five Novel Mutations

Slavomíra Mattošová MSc[1,3], Ján Chandoga MD PhD[1], Anna Hlavatá MD PhD MPH[2], Jana Šaligová MD[4] and Danka Maceková PhD[1]

[1]Institute of Medical Biology, Genetics and Clinical Genetics, Comenius University Faculty of Medicine, University Hospital Bratislava, Bratislava, Slovakia
[2]Department of Paediatrics, Comenius University Faculty of Medicine, University Children´s Hospital, Bratislava, Slovakia
[3]Department of Genetics, Faculty of Natural Sciences, Comenius University in Bratislava, Bratislava, Slovakia
[4]Children´s Faculty Hospital, Košice, Slovakia

ABSTRACT: **Background:** Gaucher disease is the most common lysosomal storage disorder and is caused by a deficiency of the enzyme glucocerebrosidase. Enzyme deficiency leads to the accumulation of undegraded substrates, mainly in cells of the monocyte/macrophage lineage, which is responsible for the clinical manifestations of the disease. To date, no study has attempted to identify the mutation spectrum of the glucocerebrosidase gene (*GBA*) in Slovak patients

Objectives: To identify mutations in 14 Slovak patients with confirmed glucocerebrosidase deficiency.

Methods: Using molecular genetics methods PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) and direct sequencing of coding region *GBA* we identified the spectrum of mutations in our patients.

Results: Five mutations (N370S, L444P, G377S, D409H and Rec*Nci*I) accounted for 75% of the mutant alleles. The remaining 25% were rare and probably individual mutations.

Conclusions: The mutational spectrum in our patients is similar to that observed in other European countries and corresponds to a Caucasian population, with N370S, L444P, Rec*Nci*I being the most common. Interestingly, mutation G377S was more frequent in our patients as compared to other published data. The C4W, L96P, H311N, 745delG and 1127_1128delTT mutations are described here for the first time in Gaucher disease, contributing to the panel of published GBA mutations.

IMAJ 2015; 17: 166–170

KEY WORDS: Gaucher disease, glucocerebrosidase, *GBA* gene, mutation analysis, novel mutations

Gaucher disease is an autosomal recessively inherited disorder caused by the deficiency of enzyme β-glucosidase (glucocerebrosidase, E.C.3.2.1.45), which cleaves the glycolipid glucocerebroside into glucose and ceramide. This leads to the accumulation of glucocerebroside in various tissues, primarily in the monocyte-macrophage cells of the liver, spleen and bone marrow [1].

The clinical presentation is extremely heterogenous. It has been classified, based on the severity of manifestations, into three different clinical forms. Type 1 (non-neuronopathic, MIM 230800) is the most common subtype and is associated with hepatosplenomegaly, bone involvement and hematological abnormalities. Type 2 (infantile, acute neuronopathic, MIM 230900) is the rarest form, and presents with early-onset severe neurological deterioration and gross organomegaly. Death usually occurs before 2 years of age. Type 3 (juvenile, chronic neuronopathic, MIM 231000) is a less acute form with childhood onset and more slowly progressive neurological disease.

The gene encoding glucocerebrosidase (*GBA*) is localized on chromosome 1q21 and spans 7.6 kb of genomic DNA. It consists of 11 exons and 10 introns. *GBA* encodes the protein of 497 amino acids. A highly homologous pseudogene (*GBAP*) is located 16 kb downstream and is 5.7 kb in length, with the same organization of exons and introns as the functional gene [2]. There is 96% sequence identity between the functional gene and *GBAP*. The presence of a pseudogene at the same locus is significant because some mutations in Gaucher disease patients are due to the recombination of *GBA* and *GBAP*. The region surrounding *GBA* contains seven functional genes and two pseudogenes [3].

More than 350 mutations have been reported in *GBA*. These include missense and nonsense mutations, small insertions or deletions that lead to frameshifts or in-frame alterations, splice junction mutations, and complex alleles carrying two or more mutations [4]. The frequencies of characteristic mutant alleles vary in different populations. The most frequent mutations are N370S and L444P. These two mutations represent 60–75% of Gaucher disease-causing alleles in most populations. In Ashkenazi Jewish patients, four mutations account for 90% of the disease alleles (N370S, L444P, 84insG and IVS2+1G>A) [5]. In Caucasian populations 60–70% of mutant alleles represent mutations N370S, L444P, Rec*Nci*I, RecTL, D409H and IVS2+1G>A [6,7]. The remaining mutations are rare. Identifying *GBA* mutations is a useful approach to establish

genotype-phenotype correlations and for genetic counseling of patients regarding clinical outcome as well as treatment recommendations.

There is significant phenotypic variation, not only among patients with the same disease type but also among patients with identical genotypes. The known mutations have been classified into three groups according to their phenotypic effect (null, severe and mild). Patients carrying at least one mild mutation (most frequently N370S) have non-neuronopathic disease (type 1), while patients carrying two severe mutations, or severe and a null mutation, usually develop neurological symptoms (type 2 and 3) [8]. Homozygosity for null allele is always associated with perinatal death [9].

In the present study we report the biochemical and molecular analysis of 14 patients with Gaucher disease from Slovakia. All patients were diagnosed by determination of glucocerebrosidase activity in peripheral blood leukocytes. The aim of this study was to identify mutations in Slovak patients with Gaucher disease and to compare the spectrum of mutations to that of other populations.

PATIENTS AND METHODS

SAMPLES

Samples were submitted to our laboratory to confirm the diagnosis of Gaucher disease, which was suspected by the patients' physicians. The diagnosis was confirmed in 14 patients (7 females, 7 males). Patients 2 and 3 are siblings. Each patient underwent biochemical analysis and mutation analysis of the *GBA* gene. DNA extraction was performed from blood samples collected into tubes with EDTA using a commercially available kit, MN NucleoSpin Blood-Mini (Macheray-Nagel, Germany). The purity of isolated DNA ranged from 1.7 to 1.9 and the yield on average was 5 µg.

BIOCHEMICAL ANALYSIS

Glucocerebrosidase activity in peripheral blood leukocytes was measured according to the method described previously [10], using fluorogenic substrate 4-methylumbelliferyl-β-D-glucopyranoside (final concentration 2 mmol/L), Glycosynth (UK) in the presence of sodium taurocholate (final concentration 0.6%). Protein concentration of the samples was determined by the Lowry method [11] using bovine serum albumin as standard.

MOLECULAR ANALYSIS

- **Detection of common mutation**

All patients were screened for common N370S and L444P mutation using polymerase chain reaction (PCR) amplification, both followed by restriction analysis. Mutation N370S was detected by mismatched PCR amplification using primers described previously [7], followed by *XhoI* digestion. Mutation L444P was detected by amplification of the corresponding DNA fragment, followed by *NciI* digestion [12].

- **Amplification of the *GBA* gene**

The exonic sequences and most intronic sequences were amplified in three fragments of 1.7–3 kb in length (exons 1–5, 5–7, 8–11), by the use of primers designed to selectively amplify the glucocerebrosidase gene and not the pseudogene sequence as described previously [13]. The reaction mixture contained 100 ng of genomic DNA, 200 µmol/L dNTPs, 2 mmol/L $MgCl_2$, 0.3 µmol/L of each primer, and 0.625 U GoTaq HotStart polymerase (Promega, USA) in a final volume of 25 µl. The amplification conditions were: initiation denaturation at 95°C for 5 min, 30 cycles of PCR with each cycle consisting of denaturation for 45 sec at 95°C, annealing for 45 sec at 60°C, extension for 3 min at 72°C, and final extension for 10 min at 72°C. Then, 10 µl of PCR products were run on 1% agarose gel to verify the amplification. Next, 15 µl of PCR products were purified using fast alkaline phosphatase and exonuclease I before sequencing. The complete coding region of the gene was sequenced on the ABI Prism 3100 Avant Genetic Analyzer using ABI Prism Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA). All identified mutations were confirmed by sequencing with both forward and reverse primers, as described elsewhere [9]. All mutations were validated by restriction digestion of the corresponding PCR products with the appropriate restriction enzyme.

EVALUATION OF A NOVEL MUTATION

Novel mutations in the *GBA* coding region were analyzed using web-based tools, PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (Sorting Intolerant from Tolerant, http://sift.bii.a-star.edu.sg/) to assess their potential pathogenicity.

RESULTS

In the 14 Slovak patients the clinical diagnosis was confirmed by biochemical and mutation analysis. According to clinical parameters, 12 patients were classified as type 1 disease, 1 patient as type 2 and 1 patient as type 3. Low residual glucocerebrosidase activity in each patient was confirmed by enzyme assay [Table 1].

MUTATION ANALYSIS

In view of these patients' clinical manifestations of disease and biochemically proved Gaucher disease, we searched for the mutational spectrum of the *GBA* gene. We identified 11 different *GBA* mutations: 7 missense, 3 frameshift mutations and 1 recombinant allele [Table 2].

Our study led to the identification of the mutation in 96% alleles (27 from 28 alleles). N370S was the most prevalent mutation, present in 9 patients (9 from 28 alleles). L444P was found in 4 patients (4 from 28 alleles) as an individual muta-

Table 1. Characteristics of patients with Gaucher disease from Slovakia

Patient no.	Gender	Glucocerebrosidase activity (nmol/h.mg proteins) (Reference values 4-12 nmol/h.mg proteins)	Genotype	Type of disease	Clinical manifestations
1	F	0.01	N370S/L444P	1	–
2	M	1.7	D409H/L444P	1	Moderate splenomegaly, mild hepatomegaly, pancytopenia
3	M	0.9	D409H/L444P	1	Severe splenomegaly, moderate hepatomegaly, hypersplenia, skeletal involvement
4	M	0.37	N370S/G377S	1	–
5	F	0.3	N370S/1127_1128delTT	1	Severe splenomegaly, mild hepatomegaly, pancytopenia, skeletal involvement
6	M	0.5	N370S/H311N	1	Severe splenomegaly, hypersplenia, mild hepatomegaly, skeletal involvement
7	M	1.0	G377S/G377S	1	Severe splenomegaly, moderate hepatomegaly, anemia, thrombocytopenia, leukopenia
8	M	0.11	G377S/L96P	3	Severe splenomegaly, mild hepatomegaly, anemia, pancytopenia, epilepsy
9	F	0.45	N370S/745delG	1	Pancytopenia
10	M	0.3	N370S/?	1	–
11	F	0.7	L444P/C4W	2	Severe splenomegaly, anemia, thrombocytopenia, myoclonic seizures with cerebral deterioration
12	F	1.0	N370S/84GG	1	Moderate splenomegaly, mild hepatomegaly, skeletal involvement
13	F	–	N370S/Rec*Nci*I	1	Mild splenomegaly
14	F	–	N370S/Rec*Nci*I	1	Mild splenomegaly

tion and in 2 patients as a part of recombinant allele Rec*Nci*I was associated with A456P and V460V (2 from 28 alleles). Mutation G377S was found in 3 patients (4 from 28 alleles). Mutation D409H was found in 2 siblings (2 from 28 alleles). Characteristics of the mutations are summarized in Table 2. We identified both mutations in all analyzed patients, except one. Five mutations (N370S, L444P, G377S, D409H, Rec*Nci*I) accounted for 75% of the mutant alleles (21 from 28 alleles). The remaining were probably individual (C4W, H311N, L96P, c.745delG, c.1127_1128delTT). Genotypes of our patients are shown in Table 1. Nine patients (64%) carried at least one copy of the N370S mutation. All patients, except one (who was homozygous for G377S mutation) were compound heterozygotes for identified mutation.

Table 2. Characteristics of mutations in Slovak patients with Gaucher disease

Mutation*§	cDNA nucleotide substitution**	Affected exon	No. of alleles	%
N370S	c.1226A>G	9	9	32
L444P	c.1448T>C	10	4	14
G377S	c.1246G>A	9	4	14
D409H	c.1342G>C	9	2	7
C4W	c.129C>G	3	1	3.5
L96P	c.404T>C	4	1	3.5
H311N§	c.1048C>A	8	1	3.5
84GG	c.84dupG	2	1	3.5
Rec*Nci*I	c.1448T>C+c.1483G>C+c.1497G>C	10	2	7
745delG	c.745delG	6	1	3.5
1127_28delTT	c.1127_1128delTT	8	1	3.5

**GBA* mutations are named according to www.hgvs.org/mutnomen
**Nucleotides are numbered from the ATG from the A of the first ATG
§Novel mutations are shown in bold
§Comparable to H311R mutation [22]

When two mutations were found in a patient, they were assumed to be on separate alleles. This was verified in patients 4, 5, 6, 10 and 11 by showing that each of their parents or children carried only one of the mutations. In the rest of the patients, their family members were not available for analysis.

Five of the mutant alleles have not been described previously. Three of them are missense mutations (C4W, L96P and H311N) and two are frameshift (c.745delG and c.1127_1128delTT). Deletion of TT at cDNA position 1127_1128 and deletion of G at cDNA position 745 leads to a frameshift with premature termination codon. It would be expected that such structurally different and shorter proteins were non-functional, and that these mutations are therefore disease-causing. In silico evaluation of the novel missense sequence variants using PolyPhen-2 and SIFT predicted that all these novel variants affect protein function. Mutation C4W was found in a patient with type 2 Gaucher disease with the L444P mutation on the second allele. Mutation L96P in exon 4 was found in combination with G377S mutation in a patient

with type 3 Gaucher disease. The remaining novel mutations were found in patients with type 1 Gaucher disease with N370S on the second allele.

DISCUSSION

The diagnosis of Gaucher disease in patients with clinical symptoms requires the proof of glucocerebrosidase activity deficiency usually in suspension of leukocytes isolated from venous blood. The glucocerebrosidase activities in all our patients were markedly reduced compared to reference values of controls. After confirming the diagnosis the next step was to identify a mutation in the glucocerebrosidase gene in each patient.

The most prevalent mutation in our patients was N370S. It was found in 32% of all mutated alleles (9/28). This frequency is somewhat lower than that observed in other central European countries [6,14,15]. Frequency of the second most prevalent mutation (L444P) was 14%. This frequency is comparable to that reported in other central European countries [6,14,15]. In Poland, due to a relatively high frequency of type 3 Gaucher disease, L444P is the most frequent mutation, followed by N370S [16]. The other frequent mutations in our patients were G377S, D409H and Rec*Nci*I. These above mentioned mutations are the most prevalent in Slovak Gaucher disease patients and accounted for 75% of mutant alleles. Mutation 84GG, one of the most common mutations in Ashkenazi Jewish populations, was found in one allele (1/28). The remaining mutations were rare and probably individual mutations. This mutation profile corresponds to that of other Caucasian populations [6,7,14,15], with the exception of G377S mutation, which was more frequent among our patients as compared to other published data from central Europe. The higher prevalence of this mutation was previously reported in patients from Portugal [17] and Brazil, in whom it is the third most frequent mutation [18,19].

The presence of the N370S mutation was always associated with non-neuronopathic disease. These results are similar to those previously reported, namely, at least one N370S allele can prevent the development of neurological signs [20]. Most of the type 1 patients were compound heterozygotes due mainly to the association of the mild N370S with another mutation. Also mutation G377S is associated with type 1 disease [21], although other reports have detected type 3 patients with one copy of G377S. Two copies of the G377S mutation generate sufficient residual enzymatic activity to prevent neurological involvement, whereas one copy combined with a severe mutation on another allele leads to type 3 Gaucher disease [19]. We report here a patient with type 3 Gaucher disease heterozygous for G377S and a novel L96P mutation. Mutation L444P may be associated with all types of Gaucher disease. In our patients, this mutation was associated with type 1 Gaucher disease in heterozygosity with N370S or D409H mutation. Another patient had novel mutation C4W on the second allele. This patient suffered from type 2 Gaucher disease.

We have identified five novel mutations among our patients (not found in the Human Gene Mutation Database). Two of the novel variants described here are certainly disease-causing mutations, as they create a frameshift and generate a premature stop codon. These mutations were found in patients with type 1 disease with mutation N370S on the second allele. The other three novel sequence variants were missense mutations. Mutation L96P was found with G377S on the second allele in a patient with type 3 Gaucher disease. Mutation H311N was found in combination with N370S mutation on the second allele in a patient with type 1 Gaucher disease. This mutation affects the same amino acid residue as mutation H311R, which most likely means that this missense change is indeed pathogenic. Mutation H311R was previously described in a patient with lethal type 2 Gaucher disease and has been classified as a severe mutation [22].

Mutation C4W was found in a female patient with type 2 Gaucher disease who presented with marked splenomegaly, anemia and thrombocytopenia, myoclonic seizures and cerebral deterioration. She died before reaching 1 year of age. Mutation C4W, which converts the first cysteine in exon 3 to tryptophan, was found with mutation L444P on the second allele. It could be predicted that this mutation has a potential deleterious effect on protein folding and disrupts the first disulphide bond within domain 1 of the X-ray structure of the glucocerebrosidase molecule, thought to be required for correct folding [23]. All cysteines except C126 are necessary for enzyme activity [1]. The loss of cysteine was previously observed in the missense mutation C16S, which resulted in type 2 phenotype [24].

The remaining undefined mutant allele in one patient can be explained by alterations outside the *GBA* coding region, in the promoter region, in untranslated or in non-coding regions. Such changes would not be detected by our approach.

The results of this study on the biochemical and molecular basis of Gaucher disease in Slovak patients contribute to the mutation spectrum of the *GBA* gene in European countries. We report five novel mutations causing Gaucher disease. We also supply data on mutations in a Central European population. Slovak patients show a profile characteristic of a European population, with the N370S, L444P and Rec*Nci*I mutations being the most prevalent. Interestingly, mutation G377S was frequent in our patients too. Genotype-phenotype correlations confirmed the previously reported protective role of N370S allele against the development of neurological complications. Identification of mutant alleles should help to establish genotype-phenotype correlations to facilitate genetic counseling and molecular analyses for families at risk. Understanding the clinical consequences of the different genotypes in our patients is critical to the establishment of procedures for monitoring and treating this disorder.

Correspondence
Dr. S. Mattošová
University Hospital Bratislava, Mickiewiczova 13, 813 69 Bratislava, Slovakia
Phone: (421-2) 572 90 192
email: smattosova@gmail.com

References

1. Beutler E, Grabovski GA. Gaucher disease. In: Sciver CR, Beauded AL, Sly WS, Vale D, eds. The Metabolic and Molecular Basis of Inherited Disease. Volume III, 8th edn. New York: McGraw Hill, 2001: 3635-8, ISBN 0-07-136321-1.
2. Horowitz M, Wilder S, Horowitz Z, Reiner O, Gelbart T, Beutler E. The human glucocerebrosidase gene and pseudogene: structure and evolution. *Genomics* 1989; 4: 87-96.
3. Winfield SL, Tayebi N, Martin BM, Ginns EI, Sidransky E. Identification of three additional genes contiguous to the glucocerebrosidase locus on chromosome 1q21: implications for Gaucher disease. *Genome Res* 1997; 7: 1020-6.
4. Hruska KS, LaMarca ME, Scott CR, Sidransky E. Gaucher disease: mutation and polymorphism spectrum in the glucocerebrosidase gene (GBA). *Hum Mutat* 2008; 29: 567-83.
5. Beutler E, Gelbart T, Kuhl W, Zimran A, West C. Mutations in Jewish patients with Gaucher disease. *Blood* 1992; 79: 1662-6.
6. Hodaňová K, Hřebíček M, Červenková M, Mrázová L, Vepřeková L, Zeman J. Analysis of the β-glucocerebrosidase gene in Czech and Slovak Gaucher patients: mutation profile and description of six novel mutant alleles. *Blood Cells Mol Dis* 1999; 25: 287-98.
7. Drugan Ch, Procopciuc L, Jebeleanu G, et al. Gaucher disease in Romanian patients: incidence of the most common mutations and phenotypic manifestations. *Eur J Hum Genet* 2002; 10: 511-15.
8. Beutler E, Demina A, Gelbart T. Glucocerebrosidase mutations in Gaucher disease. *Mol Med* 1994; 1: 82-92.
9. Stone DL, Tayebi N, Orvisky E, Stubblefield B. Madike V, Sidransky E. Glucocerebrosidase gene mutations in patients with type 2 Gaucher disease. *Hum Mutat* 2000; 15: 181-8.
10. Peters SP, Coyle P, Glew RH. Differentiation of beta-glucocerebrosidase from beta-glucosidase in human tissues using sodium taurocholate. *Arch Biochem Biophys* 1976; 175: 569-82.
11. Hartree EF. Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Anal Biochem* 1972; 48: 422-7.
12. Uchiyama A, Tomatsu S, Kondo N, et al. New Gaucher disease mutations in exon 10: a novel L444R mutation produces a new NciI site the same as L444P. *Hum Mol Genet* 1994; 3: 1183-4.
13. Koprivica V, Stone DL, Park JK, et al. Analysis and classification of 304 mutant alleles in patients with type 1 and type 3 Gaucher disease. *Am J Hum Genet* 2000; 66: 1777-86.
14. Erdős M, Hodanova K, Taskó S, et al. Genetic and clinical features of patients with Gaucher disease in Hungary. *Blood Cells Mol Dis* 2007; 39: 119-23.
15. Gorovenko NG, Oĭkhovich NV, Nedoboy AM, Pichkur NO. Determining the frequency of common mutations in the GBA gene in patients with Gaucher disease in Ukraine. *Cytol Genet* 2007; 41: 230-6.
16. Tylki-Szymanska A, Millat G, Maire I, Czatoryska B. Types I and III Gaucher disease in Poland: incidence of the most common mutations and phenotypic manifestations. *Eur J Hum Genet* 1998; 4: 334-7.
17. Amaral O, Pinto E, Fortuna M, Lacerda L, Sá Miranda MC. Type 1 Gaucher disease: identification of the N396T and prevalence of glucocerebrosidase mutations in the Portuguese. *Hum Mutat* 1996; 8: 280-1.
18. Siebert M, Bock H, Michelin-Tirelli K, Coelho JC, Giugliani R, Saraiva-Pereira ML. Novel mutations in the glucocerebrosidase gene of Brazilian patients with Gaucher disease. *JIMD Rep* 2013; 9: 7-16.
19. Rozenberg R, Araújo FT, Cox DC, et al. High frequency of mutation G377S in Brazilian type 3 Gaucher disease patients. *Braz J Med Res* 2006; 39: 1171-9.
20. Alfonso P, Cenarro A, Peréz-Calvo JI, Giralt M, Giraldo P, Pocovi M. Mutation prevalence among 51 unrelated Spanish patients with Gaucher disease: Identification of 11 novel mutations. *Blood Cells Mol Dis* 2001; 27: 882-91.
21. Amaral O, Marcao A, Sa Miranda M, Desnick RJ, Grace ME. Gaucher disease: expression and characterization of mild and severe acid beta-glucosidase mutations in Portuguese type 1 patients. *Eur J Hum Genet* 2000; 8: 95-102.
22. Stone DL, van Diggelen OP, de Klerk JB, et al. Is the perinatal lethal form of Gaucher disease more common than classic type 2 Gaucher disease? *Eur J Hum Genet* 1999; 7: 505-9.
23. Dvir H, Harel M, McCarthy AA, et al. X-ray structure of human acid β-glucosidase, the defective enzyme in Gaucher disease. *EMBO Rep* 2003; 4: 704-9.
24. Church HJ, Cooper A, Stewart F, Thornton CM, Wraith JE. Homozygous loss of a cysteine residue in the glucocerebrosidase gene results in Gaucher´s disease with a hydropic phenotype. *Eur J Hum Genet* 2004; 12: 975-8.