

Analysis of the β -Glucocerebrosidase Gene in Czech and Slovak Gaucher Patients: Mutation Profile and Description of Six Novel Mutant Alleles

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Abstract: The aim of this study was to characterize the spectrum of β -glucocerebrosidase gene mutations in Czech and Slovak Gaucher patients and to study genotype/phenotype associations. We have analyzed fifty-eight chromosomes from twenty-six type 1, two type 2, and one type 3 β -glucocerebrosidase deficient subjects by direct sequencing of PCR products. Fifty-eight mutant alleles were identified.

Seventy-eight percent of mutant alleles carried common mutations (N370S 28/58, L444P 11/58, recNciI 5/58, and IVS2(+1)A 1/58), the remaining twenty-two percent carried rare and private mutations (1263del55, 1326insT, S196P, rec(g4889–6506), 203delC, G202E, F216Y, R257X, R120W, R359Q, S107L, L444P + V460V, and D409H + T369M). Six of these alleles have not been previously described (rec(g4889–6506), 1326insT, S196P, G202E, D409H + T369M, and L444P + V460V). The most common genotypes were N370S/L444P (8/29), N370S/recNciI (5/29), and N370S/N370S (2/29).

The spectrum of the mutations is characteristic for a Caucasian (non-Jewish) population, with N370S, L444P and recNciI being the most prevalent mutations. The absence of the mutation 84insG that is frequently associated with severe bone disease may have contributed to the low incidence of severe bone disease in Czech and Slovak Gaucher subjects.

Keywords: Gaucher disease, β -glucocerebrosidase gene, mutations

INTRODUCTION

Gaucher disease (GD) is an autosomal recessive disorder of lysosomal degradation of glucosylceramide. In the overwhelming majority of patients the disease is caused by β -glucocerebrosidase (E.C. 3.2.1.45) deficiency, very rarely it is due to a deficiency of the β -glucocerebrosidase activator, saposin C. Patients usually present with splenomegaly, hepatomegaly, thrombocytopenia and often with bone disease. Patients can be divided into three clinical types on the basis of the progression of the disease and CNS involvement – type 1: non-neuronopathic (MIM 230800), type 2: acute neuronopathic (MIM 230900), type 3: subacute neuronopathic (MIM 231000). Clinical manifestation in the most prevalent type 1 is heterogeneous (1).

GD is highly prevalent in Ashkenazic Jewish population – expected incidence of GD based on the combined frequency of two most common

mutations (N370S and 84insG) is 1: 855 Ashkenazic Jewish births (2). However, a significant portion of the patients do not come to medical attention because of very mild or asymptomatic course of the disease and the estimate of incidence based on diagnosed cases is approximately 1:10 000 (2). Few data are available regarding the incidence of type 1 GD in non-Jewish populations. Type 3 GD is prevalent in Northern Sweden (Nortbotten) (3).

Human β -glucocerebrosidase gene (7.5 kb) is located on chromosome 1q21 and consists of 11 exons and 10 introns. Highly homologous β -glucocerebrosidase pseudogene lies 16 kb downstream from the functional gene (4). The region surrounding the glucocerebrosidase gene contains six other genes and a pseudogene (metaxin, metaxin pseudogene, thrombospondin 3, polymorphic epithelial mucin 1, propin 1, cotel and protein kinase clk2) (5).

More than 100 mutations, mostly single base substitutions, insertions and deletions, have been

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found in the human β -glucocerebrosidase gene in β -glucocerebrosidase deficient Gaucher patients (6,7). Whole gene deletion (8) as well as a β -glucocerebrosidase fusion gene have also been described (9). A number of complex alleles, which have presumably arisen by a crossover between the functional gene and the pseudogene, have been described (10). Common among these alleles, which are also known as recombinant or rec alleles, is recNciI, which consists of three point mutations in the coding region (L444P, A456P and V460V), and recTL, which contains the above three mutations and an additional point mutation D409H (6).

The mutation profile and mutation frequencies differ in Jewish, non-Jewish (Caucasian) and Japanese populations. In the Ashkenazic-Jewish population, the N370S mutation accounts for approximately 72% of mutant alleles. The 84insG mutation is the second most common mutation among Ashkenazi Jewish patients, accounting for 11% of mutant alleles. Mutations L444P, IVS2(+1)A, recTL, and recNciI represent another 6% of alleles. Only 11% of mutant alleles are occupied by rare or unknown mutations (6,11). No non-Jewish patients with mutation 84insG have been reported (12).

In non-Jewish (Caucasian) populations, common mutations (N370S, L444P, recNciI, recTL, D409H, and IVS2(+1)A) account for about 60–70% of mutant alleles, the rest are rare or private mutations. The L444P and N370S are the most prevalent, occupying about 20% and 15% of mutant alleles, respectively (11). In Japanese population, L444P is the most prevalent mutation, while no alleles carrying N370S have been found (13,14). Very few reports are available about the mutation analysis in Gaucher patients from Slavic populations. In the Polish Gaucher population there is a high incidence of type 3 patients with severe visceral involvement, who are homozygous for the L444P mutation (15).

The known β -glucocerebrosidase gene mutations have been divided into three groups according to their phenotypical effect (null, severe and mild mutations) (7,16). Patients carrying at least one mild mutation, most frequently N370S, have non-neuronopathic disease (type 1), while patients carrying two severe mutations or a severe and a null mutation usually develop neurological symptoms (types 2 and 3). Fetuses with two null alleles are non-viable. Although the patients sharing the same genotype tend to have similar phenotypes, there is a considerable variability which weakens the predictive value of the genotype and is well documented by the wide phenotypic range (mild to severe) observed in patients homoallelic for the N370S mutation.

The goal of the present study was to identify mutations causing Gaucher disease in β -glucocerebrosidase deficient patients from the Czech and Slovak populations, to compare the spectrum of the mutations to other populations, and to study genotype/phenotype associations.

MATERIALS AND METHODS

Subjects

Human-subject participation was approved by the local ethical committee and was in accordance with the institutional guidelines and the Declaration of Helsinki. The genotype was analyzed in twenty-six type 1, two type 2, and one type 3 Gaucher patients in whom the diagnosis of β -glucocerebrosidase deficiency was established in the Institute of Inherited Metabolic Disorders, Prague, in 1981–99. The patients in the group came from different regions of the Czech and Slovak Republics and can be thus considered representative of the Czech and Slovak populations. The patients were clinically evaluated during regular visits to our department and/or their medical records were retrospectively analyzed. In several patients the clinical data were provided by referring physicians. The

patients came from 26 families (Table 1). Five of the patients (#7, #8, #10, #28, and #29) were Slovaks and one patient (#17) was of Ukrainian origin. The rest of the patients were Czechs. Only one of the patients (#1) reported Jewish ancestry. The severity of the disease was evaluated using severity scoring index (SSI) (17). Two type 1 patients #3 and #13 and one type 2 patient #24 have deceased. Patient #13 died due to severe cardiopulmonary amyloidosis (18).

Although several of the patients had X-ray signs of bone disease and patient #21 had vertebral fractures after lifting a heavy weight, none of the patients was severely disabled due to bone disease. Patients #2, #4, #5, #11, #15, #18, #20, #21, and #22 are treated by Ceredase/Cerezyme (Genzyme).

PCR Amplifications

Genomic DNA was extracted from white blood cells or from tissues by standard methods. Coding and 5' and 3' untranslated regions of the β -glucocerebrosidase gene including intron/exon boundaries were amplified using four pairs of primers specific to the functional gene (Table 3). The primers were designed in such a way that the pseudogene was not amplified. In patients who were apparently homozygous for a mutation or when there was an obvious discrepancy between the phenotype and the apparent genotype, longer PCR products were amplified using primers G-S1 and G-A2 (product P1+2) or G-S2 and G-A3 (product P2+3+4), respectively.

Table 1. Genotypes and Phenotypes of Czech and Slovak Gaucher Patients

| No. | Sex | Age ^a | Genotype ^b | | Phenotype and notes | SSI ^c |
|-----|-----|------------------|-----------------------|--|--|------------------|
| | | | Trivial names | Systematic names | | |
| 1 | F | 51 | N370S/N370S | 1226A→G/1226A→G | mild splenomegaly, mild anemia, thrombocytopenia | 3 |
| 2 | M | 10 | L444P/L444P | 1448T→C/1448T→C | type 3, massive hepatosplenomegaly, ocular saccades, pulmonary infiltrations, growth retardation, enzyme therapy | 22 |
| 3 | F | †22 | N370S/1263del55 | 1226A→G/1263-1317del | splenectomy | - |
| 4 | M | 20 | N370S/1326insT | 1226A→G/1326TT | mild hepatosplenomegaly, pancytopenia, enzyme therapy | 11 |
| 5 | M | 11 | N370S/L444P | 1226A→G/1448T→C | moderate splenomegaly, mild hepatomegaly, enzyme therapy | |
| 6 | M | 28 | N370S/recNciI | 1226A→G/1448T→C 1483G→C 1497G→C | moderate splenomegaly, mild hepatomegaly, thrombocytopenia | 6 |
| 7 | F | 22 | N370S/recNciI | 1226A→G/1448T→C 1483G→C 1497G→C | mild splenomegaly, severe thrombocytopenia | 4 |
| 8 | F | 20 | N370S/recNciI | 1226A→G/1448T→C 1483G→C 1497G→C | mild splenomegaly, moderate thrombocytopenia sister of patient #7 | 4 |
| 9 | F | 37 | N370S/L444P | 1226A→G/1448T→C | splenectomy, mild hepatomegaly, mild thrombocytopenia | 10 |
| 10 | F | 57 | N370S/L444P | 1226A→G/1448T→C | | - |
| 11 | F | 11 | N370S/S196P | 1226A→G/703T→C | moderate splenomegaly, mild hepatomegaly, thrombocytopenia, enzyme therapy | 12 |
| 12 | M | 35 | N370S/rec(g4889-6506) | 1226A→G/1263-1317del 1342G→C 1448T→C 1483G→C 1497G→C | mild splenomegaly, mild thrombocytopenia, osteopenia, posttraumatic fractures | 9 |

Table 1—Continued

| No. | Sex | Age ^a | Genotype ^b | | Phenotype and notes | SSI ^c |
|-----|-----|------------------|-----------------------|---------------------------------------|---|------------------|
| | | | Trivial names | Systematic names | | |
| 13 | M | †50 | N370S/D409H+T369M | 1226A→G/1342G→C+ 1223C→T | splenectomy, mild hepatomegaly, severe cardiopulmonary amyloidosis (18) | 11 |
| 14 | M | 24 | N370S/L444P | 1226A→G/1448T→C | splenectomy, mild hepatomegaly | 7 |
| 15 | F | 24 | N370S/recNciI | 1226A→G/1448T→C 1483G→C 1497G→C | moderate splenomegaly, mild hepatomegaly, mild thrombocytopenia, bone pains, enzyme therapy | 11 |
| 16 | M | 58 | N370S/203delC | 1226A→G/203delC | splenectomy, mild hepatomegaly, paraproteinemia | - |
| 17 | F | 25 | N370S/N370S | 1226A→G/1226A→G | mild hepatosplenomegaly, severe thrombocytopenia, osteopenia | 9 |
| 18 | F | 44 | N370S/G202E | 1226A→G/722G→A | moderate splenomegaly, mild hepatomegaly, severe thrombocytopenia, enzyme therapy | 6 |
| 19 | F | 64 | F216Y/R257X | 764T→A/886C→T | massive splenomegaly, moderate hepatomegaly, pancytopenia, osteolytic lesions | 11 |
| 20 | F | 25 | N370S/R120W | 1226A→G/475C→T | mild hepatosplenomegaly, moderate thrombocytopenia, leukopenia, enzyme therapy | 8 |
| 21 | F | 23 | N370S/recNciI | 1226A→G/1448T→C 1483G→C 1497G→C | moderate splenomegaly, mild hepatomegaly, moderate thrombocytopenia, bone pains, osteolytic lesions, pathological fractures, enzyme therapy | 15 |
| 22 | F | 24 | N370S/R359Q | 1226A→G/1193G→A | moderate splenomegaly, mild hepatomegaly, pancytopenia, posttraumatic fractures, enzyme therapy | 8 |
| 23 | F | 37 | N370S/IVS2(+1)A | 1226A→G/IVS2(+1)G→A | moderate splenomegaly, moderate thrombocytopenia | 5 |
| 24 | M | †1 | L444P/S107L | 1448T→C/437C→T | type 2, massive hepatosplenomegaly, spasticity | - |
| 25 | F | 17 | N370S/L444P | 1226A→G/1448T→C | mild splenomegaly, mild thrombocytopenia, brother of patient #25 | 5 |
| 26 | M | 15 | N370S/L444P | 1226A→G/1448T→C | brother of patient #25 | - |
| 27 | F | 9 | N370S/L444P+V460V | 1226A→G/1448T→C+ 1497G→C | mild splenomegaly, mild thrombocytopenia | 10 |
| 28 | M | 17 | N370S/L444P | 1226A→G/1448T→C | mild hepatosplenomegaly, leukopenia, mild thrombocytopenia | 6 |
| 29 | M | 14 | N370S/L444P | 1226A→G/1448T→C | moderate splenomegaly, mild hepatomegaly, pancytopenia, pericardial effusion, brother of patient #28 | 19 |

Note. -, not done; †, age at death.

^aAge at evaluation.

^bNucleotides numbered from the first ATG (4, 21).

^cSSI-Severity scoring index.

The long-PCR technique (19) was used for all amplifications with the exception of ARMS (see below). The final volume of all PCR reactions was 100 µl. The reaction mixture contained 0.1–0.25 µg genomic DNA or cDNA, 50 mM Tris-HCl, pH 9.1, 16 mM ammonium sulfate, 150 µg/ml bovine serum albumin (BSA), 3.5 mM MgCl₂, 0.2 mM dNTPs, 20 pmol of each primer and 1 U –2.5 U KlenTaqI (AB Peptides) and 1.6×10⁻² U Deep Vent polymerase (New England Biolabs). The 35-cycle amplifications were carried out in Gene Amp 2400 (Perkin-Elmer) and PTC 200 (MJ Research) thermocyclers equipped with a heated

lid. Thermal conditions for all PCR amplifications were as follows: initial denaturation lasted 20 seconds at 95 C, each amplification cycle consisted of 5 seconds at 95 C, 15 seconds at primer-specific annealing temperature (Table 3) and 1 minute per 1 kb at 68 C for elongation. All reactions were completed by 10 minutes at 68 C. PCR products were purified by spin chromatography using Sephacryl HR-300 (Sigma) columns and ammonium acetate precipitation.

RT-PCR: Total RNA was prepared from white blood cells or cultured fibroblasts according to Chomczynski (20). Messenger RNA was reverse transcribed using SuperscriptII

reverse transcriptase (Gibco BRL) and oligo dT₁₈ primer. Full-length glucocerebrosidase cDNA was amplified using primers cDNA-S1 and cDNA-A2 (Table 3). Products from the first round of amplification served as template for two semi-nested amplifications (primers cDNA-S1 and cDNA-A1, and primers cDNA-S2 and cDNA-A2, respectively).

Sequencing

Cycle sequencing reactions contained 100–200 fmol of purified PCR products, Cy5-labeled primers and 50 mM Tris-HCl, pH 9.1, 16 mM ammonium sulfate, 150 µg/ml BSA, 3.5 mM MgCl₂ and AmpliTaqFS (Perkin Elmer) or Taqenase (Scientech). Sequencing reactions were analyzed on an automated fluorescent sequencer (ALF Express, Pharmacia).

ARMS

Amplification refractory mutation system was established for the detection of novel point mutations 1326insT, S196P and G202E. PCR primers (Table 3) were designed to discriminate between the mutant and the wild type alleles and between the gene and the pseudogene. The reaction mixture contained another pair of primers (not shown), amplifying a different region of the genome (length 800 bp), serving as an internal control for amplification. PCR reactions were carried out in the final volume of 25 µl; the reaction mixture was identical to the one described above except for Deep Vent polymerase, which was omitted.

The test series for each of these mutations also contained controls, patients' parents, and other family members, provided they were available for analysis (data not shown).

Cloning

Complex mutations, frameshifts and deletions detected by direct sequencing were verified by cloning. For verification of complex mutations PCR product P2+3+4 was digested

with XbaI and SacI, gel-purified and ligated by T4 DNA ligase into a cloning cassette prepared by digestion of pBluescript KS (Stratagene) by the same restriction enzymes. Mutations present in exons 9 and 10 were confirmed by cloning of PCR product P3 into pGEM-T vector (Promega) according to the instructions of the manufacturer. XL1-Blue cells (Clontech) were transformed with the ligation mixture and colonies containing recombinant plasmids were identified by blue/white screening. Approximately 10 white colonies were selected and the recombinant plasmids were sequenced.

Southern Blotting

Genomic DNAs from apparently homozygous patients (#1, #2, #17), from patient #12, who carries a large recombinant allele rec(g4889 – 6506) and from controls were digested with SspI (NEB), then separated on 0.8% SeaKem agarose (FMC), transferred to the nylon membrane (Zeta-Probe, Bio-Rad), UV cross-linked, and hybridized with a [³²P]-labeled full-length glucocerebrosidase cDNA probe. The radioactivity of individual bands was quantified using PhosphorImager (Molecular Dynamics) and evaluated using the software package ImageQuant (Molecular Dynamics).

Mutation Nomenclature

According to a recent proposition (7), the reference genomic (4) and cDNA sequences (21) are numbered from the first ATG codon. Commonly used trivial amino-acid-based names of the mutations are used in the text; detailed description of novel mutations is given in Table 2. In the novel recombinant complex allele rec(g4889 – 6506), the numbers in parentheses indicate the limits of the recombined region in the functional gene. Due to extreme homology between the functional gene and the pseudogene it is impossible to determine the exact points of the crossover and limits in the parentheses are given at positions where the sequences differ. Complex non-pseudogene-derived alleles are

Table 2. Novel Mutations

| Trivial name | Amino acid substitution | cDNA ^a | gDNA ^a | Exon | Detection |
|-----------------|----------------------------|--|-------------------|----------------------|-----------|
| 1326 insT | Frameshift | 1326TT | 5358 | 9 | ARMS |
| S196P | 196 Ser→Pro | 703T→C | 2914 | 6 | ARMS |
| G202E | 202 Gly→Glu | 722G→A | 2933 | 6 | ARMS |
| rec(g4889-6506) | Frameshift | 1263- 1317del 1342G→C 1448T→C 1483G→C 1497G→C | 4889- 6506 | intron 8- exon 11 | |
| D409H+T369M | 409 Asp→His 369 Thr→Met | 1342G→C 1223C→T | 5374 4856 | 9, 8 | |
| L444P+V460V | 444 Leu→Pro 460 Val→Val | 1448T→C 1497G→C | 5850 5899 | 10 | |

^aNucleotides numbered from the first ATG (4, 21).

described by a list of trivial names of mutations present on the allele (e.g., D409H + T369M).

RESULTS

Mutation Analysis

Fifty-eight mutant alleles were identified by the sequencing of PCR products from genomic DNA (Table 1). When two mutations were found in a patient, they were assumed to be on separate alleles. This was verified in patients #11, #15, #21, #23, #25, #26, #27, #28, and #29 by showing that each of their parents carried only one of the mutations. In the rest of the patients the parents were not available for analysis. In patient #12, who carries the mutation N370S and the complex allele rec(g4889–6506), and in patients #3, #4, #13, and #27, carrying genotypes N370S/1263del55, N370S/1326insT, N370S/D409H+T369M, and N370S/L444P+V460V, respectively, the alleles were verified by sequencing of cloned PCR products. Novel point mutations 1326insT, G202E, and S196P, as well as rare mutations S107L and R257X, were confirmed by ARMS.

Southern blotting did not show the presence of bands of abnormal size and the ratio of gene/pseudogene signals was identical to controls – confirming that patients #1, #2, and

#17 did not have large/whole gene deletion and that the rec(g4889–6506) allele in patient #12 did not arise by a gene/pseudogene fusion.

Seventy-eight percent of the alleles carried common mutations: N370S was found in 28 (48.3%), L444P in 11 (19%), recNciI in 5 (8.6%), and IVS2(+1)A in 1 (1.7%) alleles, the remaining twenty-two percent of the alleles carried rare and probably private mutations (1263del55, 1326insT, S196P, rec(g4889-6506), 203delC, G202E, F216Y, R257X, R120W, R359Q, S107L, L444P + V460V, D409H + T369M – each of these alleles was found only once (1.7%).

Six of the mutant alleles have not been described previously (1326insT, G202E, S196P, rec (g4889–6506), L444P + V460V, and D409H + T369M). The 1326insT is a nonsense mutation: single T insertion at cDNA position 1326 leads to a frameshift with premature termination of translation. Mutation S196P, which occurs normally in the pseudogene, has been previously described only as a part of a complex allele (22). Transition G202E affects the same amino-acid residue as severe mutation G202R (23). The rec(g4889–6506) is a complex allele due to a crossover event with the pseudogene ranging from intron 8 to exon 11. The L444P+V460V consists of two nucleotide changes present normally in the pseudogene: the common mutation L444P and the polymorphism V460V.

Table 3. PCR Primers and Products

| Primer | Nucleotide sequence (5'-3') | gDNA position ^a | cDNA position ^a | T ^{ann} (C) | PCR product | Size of PCR product (bp) | Reference |
|--------------|--|----------------------------|----------------------------|----------------------|-------------|--------------------------|---------------|
| G-S1 | CCT AAA GTT GTC ACC CAT AC | -301 | | 64 | P1 | 1896 | (39) |
| G-A1 | GGG CAG AGT GAG ATT CTG CC | 1596 | | | | | (39) |
| G-S2 | CTG ACA CTT TTC TTT GCC CT | 2165 | | 66 | P2 | 1070 | (39) |
| G-A2 | GTG ACA GAG AGA GAG ACT CC | 3234 | | | | | (39) |
| G-S3 | AAC CAT GAT TCC CTA TCT TC | 4997 | | 62 | P3 | 1768 | (39) |
| G-A3 | GGT TTT TCT ACT CTC ATG CA | 6763 | | | | | (39) |
| G-S4 | GGA GTC TCT CTC TCT GTC AC | 3130 | | 60 | P4 | 2236 | present study |
| G-A4 | TGG GAC TGT CGA CAA AGT TA | 5365 | | | | | present study |
| cDNA-S1 | TGG AAC CCC TGT GGT CTT CTC T | | -43 | 60 | cDNA-P1 | 1090 | present study |
| cDNA-A1 | TAC AGC AAT GCC ATG AAC ATA T | | 1047 | | | | present study |
| cDNA-S2 | CCC ACT TGG CTC AAG ACC AAT | | 661 | 66 | cDNA-P2 | 980 | present study |
| cDNA-A2 | GCC CAG TGC CTC CTT GAG TAT CTG | | 1640 | | | | present study |
| 1326insT-S | AAC CTC CTG TAC CAT GTG GTC GGC TGG AC | 5257 | | 58 | A-1326insT | 128 73 (pseudogene) | present study |
| 1326insT-A-M | GTA AAA CGT GTC CTT GGT GAT GTC TAC AGA | 5385 | | | | | present study |
| 1326insT-A-N | GTA AAA CGT GTC CTT GGT GAT GTC TAC AGT | 5385 | | | | | present study |
| S196P-S-M | AAT GGA GCG GTG AAT GGG AAG GTG C | 2890 | | 68 | A-S196P | 151 | present study |
| S196P-S-N | AAT GGA GCG GTG AAT GGG AAG GTG T | 2890 | | | | | present study |
| S196P-A | GTT GGG ACA CAG ATC AGC ATG GCT AAA T | 3041 | | | | | present study |
| G202E-S-M | AGG GGT CAC TCA AGG GAC AGC CAG A | 2909 | | 68 | A-G202E | 132 | present study |
| G202E-S-N | AGG GGT CAC TCA AGG GAC AGC CAG G | 2909 | | | | | present study |
| G202E-A | GTT GGG ACA CAG ATC AGC ATG GCT AAA T | 3041 | | | | | present study |

^aNucleotides numbered from the first ATG (4, 21).

Note. Long PCR products: P1+2, 3535 bp, T^{ann} = 64.3°C, primers: G-S1, G-A2.

P2+3+4, 4600 bp, T^{ann} = 60.1°C, primers: G-S2, G-A3.

T^{ann}, annealing temperature.

The D409H +T369M is a complex mutant allele consisting of the common transversion D409H and the rare transition T369M. In contrast to D409H, the T369M does not occur in the pseudogene. Partial results of molecular analysis in patient #13, who carries this allele, were published previously (18). As only exons 9–11 were studied at the time, mutation T369M was

not found and the patient was erroneously reported to have the genotype N370S/D409H.

The patients within the group showed considerable molecular heterogeneity (17 different mutant alleles and 17 genotypes), however, twenty-six patients (90%) carried at least one copy of N370S mutation. Twenty-four (83%) of them were heterozygous for the N370S

and another mutation. The most common genotypes were N370S/L444P: 8/29 patients, N370S/recNciI: 5/29 patients and N370S/N370S: 2/29 patients. The remaining 14 genotypes were found only once among the patients (Table 1).

In patient #12, who carries mutations N370S and rec(g4889–6506), the sequencing of PCR product P3 containing exons 9–11 showed homozygosity for the mutation N370S. However, when the PCR product P2+3+4 (4.6 kb) containing exons 5–11 was sequenced, the analysis of the same region revealed heterozygosity for mutations N370S, D409H, L444P, A456V, V460V, and probable presence of the 55 bp deletion 1263del55 (Figure 1). The cloning of the 4.6 kb PCR product and the sequencing of a number of clones confirmed that one allele carried mutation N370S, and the other allele contained in the coding region point mutations D409H, L444P, A456P, and V460V and the 1263del55, which occur normally in the pseudogene. This allele was not amplified by PCR with primers G-S3 and G-A3, because the functional-gene-specific primer G-S3 did not hybridize to the recombined pseudogene sequence in intron 8. No abnormal PCR products, which would suggest alternative splicing, were detected by RT-PCR. The sequencing of RT-PCR products showed the presence of expected transcripts from both alleles.

Genotype/Phenotype Correlation

The SSI scores are shown in Table 1. In five patients it was impossible to obtain complete medical records and calculate the score. In patients #2, #5 and #15, in whom long-term (> 3 years) enzyme replacement therapy led to improvement of hematological parameters and organomegaly, the score probably does not reflect the original severity of the disease. The other Ceredase/Cerezyme-treated patients, in

whom the enzyme replacement therapy was started recently, were evaluated before the therapy was initiated.

The phenotype of the patients with the genotype N370S/L444P varied from mild to moderately severe (SSI 5–19). At one extreme of the phenotypic range was patient #29, in whom the diagnosis was established at 1.5 years of age, when he already had moderate splenomegaly with thrombocytopenia and anemia. On the other extreme was patient #25 in whom mild hepatosplenomegaly with mild thrombocytopenia was noted in adolescence. Patients with N370S/recNciI genotype displayed a similar degree of variability (SSI 4–15).

Two patients homoallelic for the N370S mutation (patients #1 and #17) had a mild form of the disease.

Patients #4 and #19, who were heterozygous for a mild mutation (N370S and F216Y, respectively) and a null mutation (1326insT and R257X, respectively), each had a moderately severe phenotype. Two other patients with N370S/null genotypes, patients #23 (N370S/IVS2(+1)A) and #12 (N370S/rec(g4889–6506)) had a mild form of GD. They both had mild/moderate splenomegaly with moderate thrombocytopenia in the third decade of life with no or very slow progression of the disease in the last four years.

Patient #2, the only subject with the L444P/L444P genotype, which is regularly associated with type 3 Gaucher disease (24), had severe visceral disease with the onset in late infancy. He developed supranuclear gaze palsy during childhood. At the age of 8, pulmonary infiltrations were found, which have been recently shown to be common in patients homozygous for L444P mutation (25).

Patient #24, who had type 2 disease, carried two severe mutations (L444P/S107L). The S107L mutation was described previously only once in a type 2 patient with the same genotype as patient #24 (26).

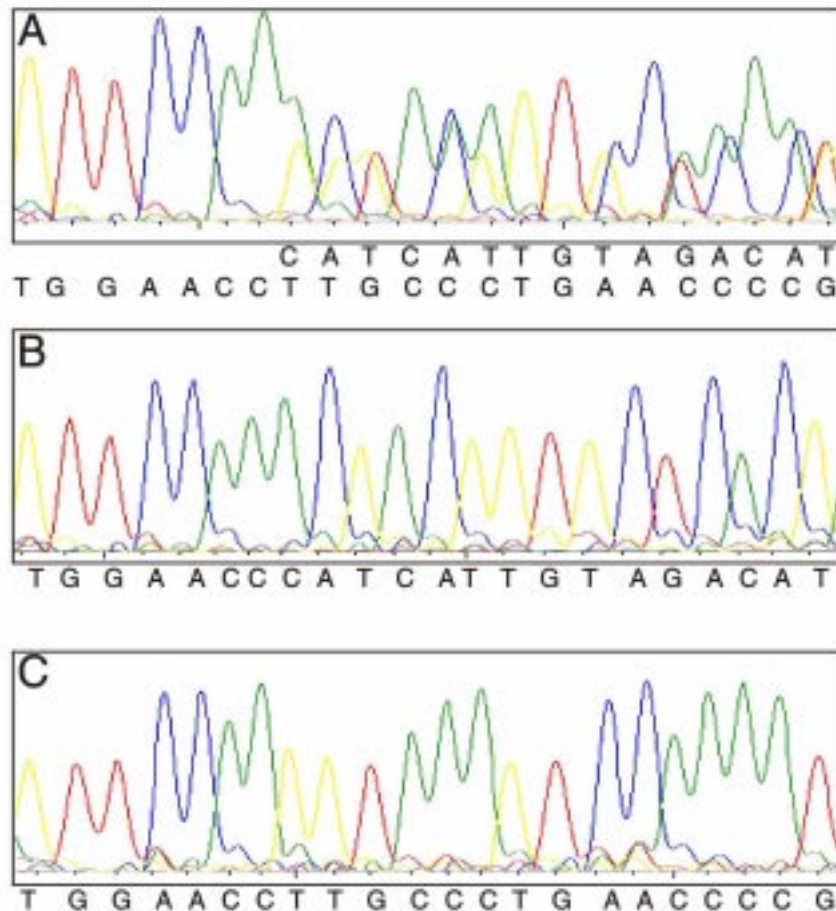


Figure 1. Direct sequencing of product P2+3+4 in patient #12 (panel A) shows “double” sequence caused by 55bp deletion 1263del55 on one allele. The sequence of both alleles was verified by cloning of the product P2+3+4 and by sequencing of multiple colonies - panel B shows the sequence of the allele carrying the 1263del55 deletion. Sequencing of product P3 (panel C) shows only normal sequence in this region, as the other allele was not amplified by functional gene specific primers.

DISCUSSION

Mutation Profile

Mutations N370S, L444P, and recNciI are the most prevalent mutations in the Czech and Slovak Gaucher patients, occupying 76% of mutant chromosomes. Rare and probably private mutations form 22% of the mutant alleles. Mutation 84insG, which appears to be specific for the Ashkenazic Jewish population (12), was not found. This mutation profile and relative frequencies correspond to a non-Jewish

(Caucasian) population (6). The mutation profile is similar to other European populations (10, 15, 27-30).

Twenty-nine patients from the Czech and Slovak populations (~10,000,000 and ~4,000,000 people, respectively) were diagnosed during the past 20 years. The local incidence of the disorder is probably higher. The observed distribution of heterozygotes and homozygotes for the N370S mutation in the patients, 24/29 and 2/29, respectively, differs from that predicted by Hardy-Weinberg equilibrium; 14/29 and 6/29, respectively. It is likely, that

asymptomatic or oligosymptomatic individuals homoallelic for N370S mutation escape ascertainment. No data about mutation frequencies in the Czech and Slovak populations are available.

Novel Mutations

Six novel mutations were found. The nonsense codons or the frameshifts that lead to a nonsense codon downstream were assumed to be the disease-producing mutations, since a truncated protein or possibly nonsense mediated mRNA decay would result from the premature termination. All novel missense mutations cause non-conservative amino-acid changes, and mutations change amino-acid residues conserved between the human and the mouse β -glucocerebrosidase cDNAs. Also, no other mutations were found in these patients within the entire coding region, intron/exon boundaries and 5' and 3' untranslated regions. This suggests that these mutations are causative.

In the rec(g4889–6506) allele, exons 9 – 11 and the flanking introns contain mutations and polymorphisms occurring normally in the pseudogene. This suggests that the allele arose due to a recombination event between the gene and the pseudogene. An allele containing in the coding region the same mutations as rec(g4889–6506) was recently described (10) – the only difference between these alleles is the inclusion of intron 8 into the recombined region in rec(g4889–6506). The rec(g4889–6506) is a null allele, as the 55 bp deletion in exon 9 leads to a frameshift and a premature termination codon.

Patient #13 carried mutations D409H and T369M on one allele. It is unclear how this double mutant allele originated. It is likely that it arose by two independent mutagenic events. As the T369M occurred in a CpG doublet, a possible explanation for the double allele is deamination of methylcytosine in exon 8 on a preexisting common D409H allele. Only several patients carrying complex non-pseudogene-derived mutations were described previously (31,32).

Although both point mutations forming L444P+V460V allele occur in the pseudogene, the allele may not have originated from a single recombination event, since in the normal pseudogene sequence there is another mutation (A456P) located between the L444P and V460V (4).

Genotype/Phenotype Correlation

The phenotypes of the patients carrying common genotypes (N370S/L444P, N370S/recNciI and N370S/N370S) were generally consistent with the usual presentation of patients with these genotypes (6). None of the patients carrying at least one copy of a mild mutation had neurological symptoms. Patients with the genotype N370S/null, who usually present with moderately severe to severe disease, had a moderately severe phenotype with the exception of patient #23 (N370S/IVS2(+1)A) and patient #12 (N370S/rec(g4889–6506)). Mutation 84insG, which is frequent in patients with severe bone disease (33) was not found among the patients. This may explain the lower incidence of severe bone disease among Czech and Slovak Gaucher patients as compared to other cohorts.

In the patients heterozygous for the very mild N370S mutation, the severity of the disease depends mainly on the mutation present at the other allele. In the type 1 patients #11, #13, #18, #20, #22 and #27, who carry N370S and a rare or private mutation (S196P, G202E, R120W, R359Q, L444P+V460V, and D409H+T369M), it is not possible to assess the severity of the mutation from this single observation. The phenotypic effect of the L444P+V460V allele is probably identical to that of isolated L444P mutation. The V460V does not change amino acid sequence and both valine codons (GUG and GUC) are frequently used in humans (34).

As in other cohorts of Gaucher patients, the patients with the same genotype tended to have similar phenotypes, but they displayed a significant clinical variability. A role for

unidentified modifier genes was postulated by different authors (35–37) to explain wider phenotypic range in the patients sharing the same genotype. Winfield and co-authors (5) speculated that a molecular lesion in genes clustered in the neighborhood of the β -glucocerebrosidase gene can contribute to the phenotype of Gaucher patients, e.g., due to a contiguous gene syndrome. Also, some of the discrepancies between phenotype and apparent genotype were shown to be due to erroneous genotype assignment (8,39). Most of all, large recombinant alleles or deletions (10) may remain undiagnosed by the currently used PCR techniques employing primers selectively amplifying the functional gene. Complex non-pseudogene-derived alleles appear to be rare; it is, however, possible that they are actually underdiagnosed. It is a common practice to assume that any two mutations found in a patient are located on different alleles and that no other mutations are present on these alleles.

In patients, who appear to be homozygous for a mutation, their parents' DNA should be tested for the presence of this mutation and/or a strategy for detection of a larger recombinant mutation or a larger deletion should be followed. At the very least, larger PCR products, which might encompass the affected region of the gene, should be prepared.

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