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Gaucher disease in Romanian patients: incidence of the most common mutations and phenotypic manifestations

Cristina Drugan*[,1], Lucia Procopciuc[1], Gheorghe Jebeleanu[1], Paula Grigorescu-Sido[2], Jane Dussau[3], Livia Poenaru[3] and Catherine Caillaud[3]

[1]Department of Biochemistry, University of Medicine and Pharmacy Cluj-Napoca, Romania; [2]Department of Paediatrics, University of Medicine and Pharmacy Cluj-Napoca, Romania; [3]Department of Genetics, Paris V University, Institut Cochim, Paris, France

Gaucher disease (GD) is an inherited glycolipid storage disorder resulting from the deficiency of glucocerebrosidase. It is the most frequent lysosomal storage disease in Romania, accounting for 70% of all lysosomal disorders diagnosed since 1997 in this country. The prevalence of six common mutations (N370S, L444P, R463C, 84GG, recNciI and recTL) and their phenotypic impact were studied in 20 type 1 GD patients of non-Jewish origin. Mutation analysis identified 77.8% of the GD alleles. The N370S mutation had the highest prevalence (50%), followed by the L444P (22.2%) and the recNciI (5.6%) alleles. Mutations R463C, 84GG and recTL have not been found in our patients. Rare or novel mutations likely accounted for 22.2% of the disease-producing uncharacterised alleles. Our study indicates a high prevalence of type 1 among Romanian GD patients. Clinical phenotype and disease severity were evaluated according to the standardised severity score index. Genotype–phenotype correlations were similar to those reported for other Caucasian non-Jewish populations. The absence of neuronopathic disease in patients presenting at least one copy of the N370S allele was confirmed, but the relative mildness of N370S homozygotes was not a constant feature among our patients. The presence of the L444P or of uncharacterised sporadic mutations was always associated with severe clinical manifestations, even in compound heterozygotes with the N370S allele. A large degree of phenotypic variability was observed in patients displaying the same genotype. The particularities of genotype–phenotype correlations may suggest the impact of other genetic or non-genetic factors on the clinical picture.
European Journal of Human Genetics (2002) **10**, 511–515. doi:10.1038/sj.ejhg.5200845

Introduction

Gaucher disease (GD) is an autosomal recessive sphingolipidosis caused by a deficiency of the lysosomal hydrolase acid β-glucosidase (glucocerebrosidase, E.C. 3.2.1.45). It results in a progressive accumulation of glucosylceramide in the lysosomes of macrophages, leading to hepatosplenomegaly, anemia, thrombocytopenia and various skeletal complica-

*Correspondence: C Drugan; Department of Biochemistry, University of Medicine and Pharmacy, Str Splaiul Independenei 4/7, 3400 Cluj-Napoca, Romania. Tel: +40-95-617634; Fax: +40-64-193847;
E-mail: cdrugan@umfcluj.ro

tions. GD has a striking phenotypic variability, with respect to age of onset, progression and severity. However, three main clinical types have been delineated according to the absence (type 1, non neuronopathic) or the presence (type 2, acute neuronopathic and type 3, subacute neuronopathic) of neurological involvement.[1] To date, more than 120 disease-producing alleles have been described, but only a few point mutations (N370S, 84GG, L444P, IVS2+1g>a, D409H) and complex alleles (resulting from genetic rearrangements between the functional gene and the closely located pseudogene) occur with significant frequencies in

GD patients.[2] The prevalence of different mutations has been extensively studied and some useful genotype – phenotype correlations have been inferred.[3,4] Although the predictive value of these correlations is generally limited, their study in different ethnic groups had a significant impact on genetic counselling and on the better understanding of the molecular aspects of GD.

We report here the first study of mutations causing GD in Romanian patients. The biochemical diagnosis of GD was introduced in 1997 in this country, based on the measurement of glucocerebrosidase activity in peripheral blood leukocytes. Since then, GD has been the most frequent (70%) lysosomal storage disorder diagnosed in our laboratory. Twenty patients belonging to 18 different families were tested for the presence of six known mutations (N370S, L444P, R463C, 84GG, recNciI and recTL). The aim of this study was to establish the most frequent mutations in our group of patients and to analyse their phenotypic impact.

Materials and Methods
Patients
Patients originated from various regions of Romania. All of them declared a non-Jewish ethnic origin. Clinical evaluation included haematological tests, investigation of liver and spleen function, investigation of the presence and the extent of organomegaly, ophthalmologic examination and evaluation of skeletal and neurological involvement (clinical and electro-encephalographic examination). The degree of clinical severity was established according to the severity score index (SSI) proposed by Zimran *et al*.[5] As enzyme replacement therapy is just starting in Romania, the clinical status of the patients reflected the natural course of GD, prior to any specific treatment. Measurement of glucocerebrosidase activity in leukocytes was performed using the artificial substrate 4-methylumbelliferyl-β-D-glucopyranoside.[6]

Detection of mutations
High molecular weight genomic DNA was extracted from peripheral blood leukocytes according to standard methods.

Mutation N370S was detected by mismatched PCR amplification, as previously described,[7] with minor modifications. Primer sequences were extended (5′-GTCTCTTT-GCCTTTGTCCTTACCCTCGA-3′ for the upstream primer and 5′-ACTGTCGACAAAGTTACGCACCCAAT-3′ for the downstream primer) yielding a more specific amplification of a 120 bp fragment bearing the N370S mutation. The base changed to create an *Xho*I site is underlined. Mutation 84GG was also studied by mismatched PCR amplification, as previously described.[8]

Mutations L444P and R463C were studied by selective amplification of the corresponding DNA fragment, followed by *Msp*I digestion, according to the method previously described.[9] The recombinant alleles recNciI (including mutations L444P, A456P and V460V) and recTL (including mutations D409H, L444P, A456P and V460V) were analysed by sequencing of the amplified fragments bearing the L444P substitution. PCR products were purified using the QIAquick PCR purification kit (Qiagen). The sequencing reactions were performed on an automated ABI 373A DNA sequencer with the Big Dye Terminator Ampli Taq FS kit (ABI), according to the manufacturer's recommendations.

Biochemical and molecular testing of the family members (siblings and both parents) were conducted for each index case, in order to identify possible asymptomatic relatives and to ensure the correct genotyping of homozygotes.

Results
Patient characteristics
The studied group consisted of 20 non-Jewish Gaucher disease patients (14 females and six males, with ages ranging from 2 to 53 years), displaying a marked reduction of β-glucocerebrosidase activity. Their most important clinical features are summarised in Table 1. Forty-five per cent (nine out of 20) previously underwent splenectomy, whereas eight of the remaining patients (40%) had splenomegaly. Hepatomegaly was observed in 17 patients (85%). Thrombocytopenia was present in 10 patients (50%) and anemia in 16 patients (80%). Bone abnormalities of different grades have been found in 95% of the patients and included Erlenmeyer flask deformities (90%), chronic bone pain (65%), osteoporosis/osteolysis (30%), pathologic fractures/hip replacement (20%). All the patients were classified as having the non-neuronopathic (type 1) form of the disease, according to the absence of any signs of neurological involvement.

Mutation analysis
Screening for the most frequent mutations allowed the identification of 77.8% of the mutant alleles. Among the six common mutations tested, only three were found in this study: N370S (50%), L444P (22.2%) and recNciI (5.6%). Sporadic uncharacterised mutations accounted for 22.2% of the mutant alleles. Mutations R463C, 84GG and recTL were not found in our patients. Since our group of patients included two pairs of siblings, the frequencies were calculated on a total number of 36 alleles.

Genotypes of the patients are presented in Table 1. Most of them comprised the N370S mutation, either in a homozygous state (three patients), or in association with the L444P or recNciI alleles (eight patients), or with an uncharacterised mutation (seven patients). Only one patient was homozygous for the L444P mutation and one was a compound heterozygote for the L444P and an unknown mutation.

Genotype – phenotype correlations
The majority of the patients, whatever their genotype, exhibited various degrees of haematological and skeletal

Table 1 Genotypes and clinical features of Romanian Gaucher patients

No	Sex	Age*	Genotype	Splenectomy/ age**	Clinical type	Severity score index	Skeletal involvement	Other clinical particularities
1	F	23	N370S/N370S	No	1	3	Absent	Moderate thrombocytopenia
2	F	26	N370S/N370S	No	1	14	Diffuse osteoporosis Erlenmeyer flask deformities Chronic pain	Severe thrombocytopenia and anaemia Massive hepatosplenomegaly
3	F	42	N370S/N370S	No	1	13	Erlenmeyer flask deformities	Severe thrombocytopenia and anaemia Moderate hepatosplenomegaly
4	M	4	L444P/L444P	Yes/4	1***	16	Erlenmeyer flask deformities Chronic pain	Growth retardation Severe anaemia Massive hepatomegaly
5	F	9	N370S/L444P	Yes/9	1	15	Osteolysis Erlenmeyer flask deformities Chronic pain	Severe anaemia Massive hepatomegaly
6	M	13	N370S/L444P	Yes/13	1	17	Erlenmeyer flask deformities Chronic pain Pathological fractures	Growth retardation Moderate hepatomegaly
7	F	18	N370S/L444P	No	1	14	Osteolysis Erlenmeyer flask deformities Occasional pain	Growth retardation Severe anaemia Massive hepatosplenomegaly
8	F	25	N370S/L444P	No	1	16	Erlenmeyer flask deformities Chronic pain	Moderate hepatosplenomegaly Severe thrombocytopenia
9	F	31	N370S/L444P	Yes/13	1	20	Erlenmeyer flask deformities Chronic pain Avascular femoral necrosis Femoral osteomyelitis Repeated pathologic fractures	Severe anaemia Massive hepatomegaly
10	F	17	N370S/recNciI	No	1	14	Osteolysis Erlenmeyer flask deformities Chronic pain	Severe cytopenia
11	M	19	N370S/recNciI	No	1	14	Erlenmeyer flask deformities	Severe cytopenia
12	M	26	N370S/recNciI	No	1	13	Erlenmeyer flask deformities Chronic pain	Moderate thrombocytopenia Massive hepatosplenomegaly
13	F	11	N370S/?	No	1	12	Osteolysis Erlenmeyer flask deformities Occasional pain	Severe thrombocytopenia and anaemia Moderate hepatosplenomegaly
14	F	10	N370S/?	No	1	11	Erlenmeyer flask deformities Occasional pain	Severe thrombocytopenia and anaemia Moderate hepatosplenomegaly
15	M	33	N370S/?	Yes/28	1	14	Erlenmeyer flask deformities Chronic pain	Moderate hepatomegaly
16	F	40	N370S/?	Yes/12	1	20	Avascular femoral necrosis Erlenmeyer flask deformities Chronic pain	Severe anaemia Massive hepatomegaly
17	M	41	N370S/?	Yes/6	1	20	Bilateral hip replacement Chronic pain	Severe anaemia Moderate hepatomegaly
18	F	41	N370S/?	Yes/8	1	21	Erlenmeyer flask deformities Chronic pain Repeated pathologic fractures	Severe anaemia Massive hepatomegaly
19	F	53	N370S/?	Yes/16	1	12	Erlenmeyer flask deformities Chronic pain	Severe anaemia Mild hepatomegaly
20	F	2	L444P/?	No	1***	13	Erlenmeyer flask deformities	Failure to thrive Massive hepatosplenomegaly Severe cytopenia

*The age (expressed in years) corresponds to the moment of enzymatic diagnosis and has not been strictly considered as a criterion of disease severity, because all patients were formally diagnosed after 1997, when the enzymatic assay became available. Therefore, this parameter cannot reflect the real age at which the diagnosis would have been established. **The age at splenectomy may be anterior to the age at which the enzymatic diagnosis was established. ***No signs of neurological involvement were detected in patients no 4 and 20 at the age of 8 and 4 years old, respectively. Patients no 1–2 and 10–11 are siblings. The severity score index was calculated according to Zimran *et al*[5].

involvement, as reflected by the SSI values ranging between 11 and 21. The most severe bone complications (multiple fractures, hip replacement) were found in two patients carrying the N370S mutation, in asso-ciation with the L444P or with an uncharacterised mutation.

One patient homozygous for the N370S mutation (no. 1) was nearly asymptomatic (SSI=3), even though her sister

(no. 2) exhibited hepatosplenomegaly, pancytopenia and bone involvement (SSI=14). The two patients carrying the L444P mutation, either in a homozygous state, or in association with an unknown mutation, had no neurological involvement, either at the time of diagnosis, or at a subsequent re-evaluation at the age of 8 and 4, respectively.

Discussion

The distribution of mutant alleles identified in our patients was compared with data originating from different populations. The frequency of the N370S allele in Romanian GD patients (50%) was higher than that reported in other non-Jewish European populations,[10,11] except Portuguese patients.[12] The second most prevalent mutation, L444P, was responsible for 22.2% of the mutant alleles, a frequency comparable to that reported for other non-Jewish populations (25%).[2] The absence of the 84GG mutation is not surprising, as this mutation is considered to be specific for Jewish GD patients.[13] The distribution of common mutations in Romanian GD patients was close to that established for Czech and Slovak patients,[14] but different from another East European country, Poland,[15] where the L444P substitution is the most prevalent mutation, due to a relatively high frequency of type 3 disease. Our results show some similarities with other non-Jewish East-European populations,[14,15] but also indicate a high prevalence of common mutations (77.8%), which may be related to the higher ethnic homogeneity of our population.

The presence of two siblings homozygous for the N370S allele with markedly different clinical presentation supports the observed intra-familial heterogeneity of GD patients, indicating that other genetic or non-genetic factors may contribute to the clinical picture of the disease.[16] The asymptomatic sibling was diagnosed by family investigations following the identification of the index case, suggesting that other asymptomatic N370S homozygotes may exist in our population, but do not come to medical attention, as previously shown by Zimran et al [17] in the Ashkenazi Jewish population.

In our study, the presence of the N370S mutation was always associated with non-neuronopathic disease. These results are similar to those previously reported, concerning the protective role of at least one N370S allele with respect to the development of neurological signs.[18] However, patients who were compound heterozygotes for the N370S allele and an unknown mutation displayed a large degree of clinical variability (SSI values ranging between 11 and 21). This group included the patients who were most severely affected by the skeletal and haematological complications of GD. These data are different from those previously described in other non-Jewish populations, indi-

cating a correlation between at least one N370S allele and a milder course of the disease.[19]

The L444P mutation may be associated with all types of Gaucher disease. In our study, most of the patients carrying the L444P mutation were compound heterozygotes for this allele and the N370S mutation. A constant feature was the presence of severe skeletal involvement (SSI values between 13 and 20) and a more severe course of the disease, including delayed growth in children.

No neuronopathic involvement has been demonstrated in the patient homozygous for the L444P allele, either at the time of diagnosis or at a subsequent follow-up examination at the age of 8, which included an electro-encephalographic and ophthalmologic evaluation. However, as an electro-oculographic recording of eye movements was not available, the presence of subclinical oculomotor abnormalities or the future evolution towards the subacute neuronopathic type 3 GD cannot be excluded in this patient, although homozygosity for the L444P mutation has already been found associated to type 1 GD.[20]

One patient was heterozygous for the L444P allele and an unknown mutation. This patient died at the age of 4, following complications of massive hepatosplenomegaly, but again no neurological involvement could be demonstrated. The clinical picture may suggest a possible type 3b variant, in which death may have occurred before the involvement of the central nervous system became detectable.[21]

Our study provides an insight into the molecular bases of GD in Romanian patients. The distribution of mutant alleles and the frequency with which particular genotypes were encountered displayed some specific particularities, which may be related to the ethnic characteristics of our population. Genotype–phenotype correlations confirmed the previously reported 'protective' role of the N370S allele against the development of neurological complications and also indicated a more severe phenotypic impact of the L444P allele and of some sporadic, uncharacterised, mutations. Phenotypic variation was described among patients with identical genotypes, confirming that other genetic, environmental or developmental factors may contribute to the clinical expression of GD. Understanding the natural history of the disease and the clinical consequences of the different genotypes in our patients is critical to the establishment of procedures for monitoring and treating this lifelong disorder.

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