RESEARCH ARTICLE

GBA mutations in Gaucher type I Venezuelan patients:

ethnic origins and frequencies.

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Running title: *GBA* mutations in Venezuelan patients.

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Abstract

Gaucher disease (GD), the most frequent lysosomal storage disease, is caused by heterogeneous mutations in the locus encoding for glucocerebrosidase (*GBA*). It is an autosomal recessive disorder with different phenotypes; the most frequent of which is the nonneuronopathic or type 1, prevalent worldwide. To date, more than 430 mutations have been described, but their frequency distribution varies in different populations, with four of them: N370S, L444P, IVS2+1G>A and 84insG being the frequent ones. In Venezuela 20

unrelated index cases with GD type I were assessed for *GBA* mutation detection and for their in-phase haplotype identification, to gather genetic epidemiological data on the disease in the country and of its eventual ethnic origin. Ten missense mutations and 2 complex alleles were identified. The most frequent were N370S (42.5%), L444P (20%), IVS2+1G>A (10%) and R48W (5%); mutations R120W, P245H, H311R, R496H, W36X and R433G were carried by a single chromosome each. Three geographic foci were identified, displaying mutation heterogeneity. N370S had multiple genetic origins, different from the Ashkenazim's; a single common remote ancestor for this mutation in the country was dismissed, according to the haplotype analysis. All mutations have a likely European Caucasoid descent.

Introduction

Gaucher disease (OMIM#230800), the most common of lysosomal storage diseases, is an autosomal recessive disorder resulting from deficit or lack of β-glucosidase activity. The glucocerebrosidase (acid- β glucosidase, glucosyl-ceramidase, EC.3.2.1.45), a lysosomal glycoside hydrolase, cleaves the β-glucosidic linkage of glucosylceramide, a normal intermediate of glycolipid catabolism (Beutler and Grabowski, 1995). The abnormal function leads to accumulation of the substrate (glucocerebroside) in cells of the macrophage-monocyte system.

The disease is highly heterogeneous and has been classified into three types based on the presence and rate of progression of neurologic manifestations. The most common (95% of cases) nonneuronopathic Gaucher disease type 1 (OMIM#230800) is characterized by the presence of hepatosplenomegaly, pancytopenia and skeletal involvement, without neurological manifestations, and a variable age of onset of symptoms; bone marrow infiltration by 'Gaucher cells' is typical. In the type 2 disease (OMIM#230900) or acute neuronopathic, the patients present with a severe hepatosplenomegaly and progressive

neurologic deterioration, being usually fatal within the first 2 years of life. Type 3 (OMIM#231000) or chronic neuronopathic disease has a heterogeneous presentation with variable neurological and visceral involvement, onset in childhood or in adulthood; almost all patients survive until adulthood (Vellodi *et al*, 2001).

The gene encoding glucocerebrosidase (*GBA*; 1q21) encompasses 7 kb with 11 exons, producing a cDNA of approximately 2.5 kb; the mature protein has a 39-amino acids signal peptide. *GBA* has a highly homologous pseudogene (*psGBA* with 96% identity), located 16 kb downstream (Horowitz *et al,* 1989) with the same organization of exons and introns as the functional gene, but carrying large deletions in introns 2, 4, 6 and 7 which correspond to *Alu* elements. These highly homologous sequence and physical proximity lead to gene conversion and crossing over events between *GBA* and *psGBA*, producing mutated complex alleles.

To date, around 437 different mutations at the *GBA* gene have been registered in the human mutation database at Cardiff, UK (www.hgmd.cf.ac.uk/ac/). Nevertheless, four mutations (N370S, L444P, 84insG, IVS2+1G>A) account for 90% of Ashkenazim patients but for only 50-60% of cases in other different populations worldwide, many of them being compound heterozygotes.

In this study, 20 Venezuelan independent families with Gaucher disease type I ascertained along 20 years were assessed, to identify *GBA* mutations and to study genetic epidemiological features of the disease in the country. The most frequently found, mutation N370S had a heterogeneous origin, different from the Ashkenazim's, according to the haplotype analysis.

Materials and Methods

Sample

A total of 20 genetically unrelated index cases with biochemical and clinical diagnosis of Gaucher disease, who were referred to the Human Genetics Laboratory (HGL) at the Venezuelan Institute for Scientific Research (IVIC) and to the Hematology Service at the Miguel Pérez Carreño Hospital (Caracas) were included in the study. Two additional affected family members (a sister and an aunt) of one of the families (#13, Table 1) were also included in the study.

The diagnosis of Gaucher disease was established by the clinical manifestations and a diminished β-glucosidase activity. The enzymatic activities were measured either at the HGL, according to protocols by Peters *et al* (1977) and Daniels *et al* (1981), or at CENTOGENE, Germany, using mass spectrometry.

The geographic origin of each family was established by recording the precise place of birth of the remote ancestors (grandparents and great-grandparents).

For the molecular analyses, a 5ml blood sample was collected, EDTA anti-coagulated, and DNA was extracted by a saline method (Lahiri & Nurnberger, 1991).

Written voluntary informed consent was obtained from all family members, according to the bioethical institutional guidelines.

DNA analyses

Mutation detection

A two stage PCR method was used to selectively amplify the glucocerebrosidase functional gene, but not the pseudogene. In the first stage, only the functional gene was amplified in three large amplicons (between 1681 bp to 2971 bp). In the second stage, the first round PCR products were used as templates for the amplification of each and all the exons of the *GBA*

gene (nested PCR). Primers were previously reported by Stone *et al* (2000). The Genbank reference sequence accession number was NM_000157.3.

Single strand conformation polymorphism (SSCP) analysis of each exon was performed as previously reported (Paradisi and Arias, 2010), and those showing any abnormal migration pattern were sequenced at Macrogen, Seoul, Korea.

Polymorphisms used to construct haplotypes

Two intragenic single nucleotide polymorphisms (SNP) at intron 6 and intron 7, g.4813G>A [rs762488; NM_000157.3: c.762-180G>A] and g.5470G>A [NM_000157.3: c.999+240G>A], and 2 microsatellites (5GC3.2 and ITG6.2) located in the flanking regions of the gene were used to construct the haplotypes in phase with the mutations. To establish the phases, genotypes of each marker in carriers and noncarriers family members were established, and segregation analysis of the allele transmission from parents to descendants was assessed, in each polymorphic site.

Primers were previously reported by Lau *et al* (1999) and Rodríguez-Marí *et al* (2001).

Alleles at the SNPs were detected by the restriction enzymes (*Pvu*II for g.4813 G>A and *Bsu*36I for g.5470 G>A); microsatellites 5GC3.2 (dinucleotide repeats CT) and ITG6.2 (tetranucleotide repeats AAAT) were analyzed in a 10% and 8% polyacrylamide (acrylamide-bisacrylamide, 39:1) gel electrophoresis respectively. Some samples were Sanger sequenced to confirm the actual allele sizes.

Results

The main phenotypic and genotypic data of the studied index cases are shown in Table 1. All the patients had a type I form of the disease (nonneuronopathic), with a highly variable age of onset and common clinical manifestations.

Almost all patients had splenomegaly (94.7%) and hepatomegaly (84.2%); more than a half had thrombocytopenia (52.6%) and 42.1% had anemia. Bone crisis occurred in 10.5% of cases. In 45% of patients, the diagnosis was made in the first decade of life, in 20% in the second decade, and in 35% during the third, fourth or fifth decades; 14 out of 20 were female. All the patients are receiving enzymatic replacement therapy.

GBA analysis

Complete coding region and intron-exon boundaries of the *GBA* gene were screened; 10 different mutations and 2 complex alleles in 40 studied chromosomes were identified, all of them previously reported in different populations.

The most frequent mutation was N370S, carried by 17 chromosomes (42. 5%), followed by L444P (20%), presenting as a complex allele or as a single allele (5%); IVS2+1 G>A was present in 10% of chromosomes, while the R48W mutation was found in 5%.

Mutations R120W, P245H, H311R, R496H, W36X and R433G showed low frequencies, being carried by a single chromosome each one. All of these mutations cause deleterious effects on the protein structure, with Polyphen scores of 1.0 (P245H, H311R and R433G), 0.999 for R120W and 0.488 for R496H. Mutation W36X causes a severely truncated protein. 95% of index cases were compound heterozygotes.

All the mutation names refer to the processed protein, not including the 39-residue signal peptide.

Haplotype analysis and geographic distribution

Only seven out of 20 families (35%) had geographic aggregation, with remote ancestors coming from three different geographic foci (Arias, 1994) in Zulia state (3 independent families), in Lara state (3 independent families) and in Yaracuy state (1 family); the remaining 65% of families ancestors were scattered across the country (Table 1).

Within the geographic foci there was mutation heterogeneity, with 2 different mutations in the Zulia state focus (center in La Cañada), 5 mutations in the Lara state focus (center in Barquisimeto) and 2 mutations in the Yaracuy state focus (center in San Felipe), suggesting different genetic origins for Gaucher disease in each one.

In the Zulia state focus, the three unrelated index cases (# 8, 9 and 13, Table 1) were compound heterozygotes for mutations IVS2+1G>A and N370S, which were both in phase with the same 314;G;222;G haplotype in the three families.

Family of index case # 13 contained two compound heterozygous sisters (N370S/IVS2+1G>A) and an affected maternal aunt carrying N370S plus another non identified change; the paternal one (IVS2+1G>A) had a recent central European Ashkenazim origin; but the maternal N370S frequent in the focus, is not a Jewish mutation according to its in-phase haplotype, being apparently only identical by nature (IBN).

Index case #7 also carried IVS2+1G>A/N370S mutations with the same in-phase haplotypes, but her remote ancestors origins were different, coming from two far apart states (Sucre state and Falcón state, at the oriental and occidental regions of the country).

This finding strongly suggests that in most cases those mutations both in non Jewish and in Ashkenazim carriers might be likely also identical by descent (IBD), going back in their ancestry much earlier than one thousand years.

In the Lara state focus, 3 different mutations and 2 complex alleles were present; the three unrelated index cases (# 3, 4 and 6, Table 1) were compound heterozygotes [R48W]/[R120W], [N370S]/ [L444P+A456P+V460V], and [N370S]/[E326K+L444P]. Interestingly, the N370S mutation had the same in-phase 314; G; 222; A haplotype, shared by the two independent families, and different from that found in the Zulia state focus, supporting thus different origins between foci for N370S. The R48W, a very infrequent mutation worldwide, was found in two foci (Yaracuy and Lara states, 90 Km afar: index cases # 2 and 3, Table 1) with the same in- phase haplotype 318; G; 222; A, suggesting a common but very remote ancestor.

Table 2 shows the haplotypes in phase with the twelve detected mutations. The most frequent mutation N370S was in phase with four different haplotypes, as well as the second most frequent L444P (alone and in complex alleles); in three instances, N370S and L444P had the same haplotypes, which were also very frequent in patients: 314; G; 222; G (37.5%), 314; G; 222; A (22.5%) and 318; G; 224; G, (7.5%) but not so in controls. Infrequent mutations had a unique in-phase haplotype, except for R48W, as already mentioned.

Discussion

Gaucher disease (GD) is the most frequent disorder among lysosomal storage diseases, although it is infrequent in populations worldwide. In Venezuela, the "Venezuelan Association of patients with lysosomal diseases" (AVEPEL) has 81 registered independent index cases coming from the country at large, all receiving enzymatic replacement therapy provided by the Governmental Social Security Agency. Thus, the disease prevalence in Venezuela can be estimated as 1:77000 families if all existing cases had been detected, calculated as previously reported (Paradisi et al, 2015), which is similar to the figure quoted in general worldwide populations, around 1.3:100000 inhabitants, excluding the Ashkenazi

Jewry of eastern and central European ancestry, in which the prevalence is of 1:500 to 1:1000 (Orphanet Reports Series, at www.orpha.net).

In the 20 independent index cases the mutation detection rate was 92.5%. A genotype-phenotype correlation could not be established, since there were 13 different genotypes and all but one index cases were compound heterozygotes for different mutations. The exception were the IVS2+1 G>A/N370S carriers, which accounted for 20% of the sample, between them however, there was no clear genotype-phenotype correlation (Table 1).

Mutations N370S and L444P are the most common mutations worldwide; jointly they represent between 50 and 62% of the *GBA* mutations in all populations, except among the Ashkenazim, in which they account for 93% of the mutant alleles (Koprivica *et al* 2000); in the Venezuelan patients, its joint frequency was 62.5%. N370S is virtually absent in Mongoloid populations.

Mutation N370S has been reported to cause relatively minor changes in the glucocerebrosidase structure and therefore, in its catalytic activity (Dvir *et al*, 2003). It is located at the interface of domains II and III, too far from the active site to participle directly in catalysis. Thus, its phenotypic effect tends to be moderate, producing always a type I disease with mild clinical manifestations and an older age at diagnosis, even when in heterozygosity (Charrow et al, 2000), as was observed in our patients (Table 1). Polyphen analysis classifies its pathogenicity as possibly damaging, with a score of 0.607.

As mentioned mutation N370S is the most frequent in Ashkenazim, due to a founder effect and a genetic drift phenomena. In such populations, it shows a strong allelic disequilibrium with the 222 bp and 318 bp alleles of microsatellite markers 5GC3.2 and ITG6.2 (Zimran *et al*, 1990; Amaral *et al*, 1997; Cormand *et al*, 1998; Rockah *et al,* 1998), as well as with the A

allele at intron 6 (g.4813 G>A, which suppress the restriction site for the *Pvu*II enzyme known as the Pvu1.1⁻ haplotype); and the G allele at g.5470 G>A (c.999+240G>A). Thus, haplotype 318; G; 222; A (ITG6.2; g.5470 G>A; 5GC3.2; g.4813 G>A) is almost always in phase with the Ashkenazi N370S mutation. In Venezuelan patients, the N370S had at least 3 different in-phase haplotypes: 314; G; 222; G (47.1%), 314; G; 222; A (35.3%), 318; G; 224; G (11.8%) and 318; G; (-); (-) (5.8%) (Table 2). The in-phase haplotype with the highest frequency (314; G, 222; G), has been found also by Wilches *et al* (2006) in Colombian N370S carriers; Colombian and Venezuelan populations share a very similar demographic history, suggesting a plausible common remote origin for the mutation carrying this haplotype in phase. On the other hand, haplotypes in phase with this N370S "Venezuelan" mutation suggest that the origin is different from the Ashkenazim's in most cases, and that there is not a single common ancestor for the mutation in the country. In Venezuela, most of the N370S alleles are distributed in the northwestern region, showing geographic aggregation in two foci: La Cañada (Zulia state) and Barquisimeto (Lara state). Nevertheless, in-phase haplotypes were different between foci (314; G; 222; G and 314; G; 222; A) respectively, suggesting separate origins for the N370S in each focus, but a common source within each one.

The L444P occurs in the hydrophobic core of the Ig-like domain (domain II), causing protein instability due to disruption of the hydrophobic core and altered folding of the domain (Beutler and Kuhl, 1986; Dvir *et al,* 2003); its Polyphen score is 0.938, suggesting a strong mutation effect on the protein structure. This mutation in homozygosis only is associated with the chronic neuronopathic form of Gaucher disease. It is the second most frequent mutation (after N370S) in Ashkenazim, Caucasoids (Stinermann *et al*, 2012) and also in the Venezuelan studied patients, but not in East Asians Caucasoids or SubSaharan Africans.

Two haplotypes in phase with the L444P mutation as a single allele (318; G; 222; G and 318; G; 224; G), and two other when in complex alleles (Table 2) were found in the index cases. Tuteja *et al*, (1993) demonstrated that several haplotypes are in phase with this mutation and suggested multiple ancestral origins for it, since codon 444 could be a hot spot for mutations. The mutation has been associated with the $Pv1.1^{-}$ haplotype in Jewish populations (Rodriguez-Marí *et al*, 2001). In Venezuelan patients, all chromosomes carrying L444P were in phase with allele G (haplotype $Pv1.1^{+}$), discarding thus a possible remote Jewish origin for it. Furthermore, haplotype diversity suggests multiple origins of L444P in the country.

Mutation IVS2+1G>A was identified in 4 patients (4 chromosomes, 10%). The change produces aberrantly spliced mRNAs, missing exon 2, and severely impaired catalytic activity (He and Grabowski, 1992). This mutation represents 1-4% of patients with Gaucher disease, in the "general" population, ordinarily associated to the N370S mutation, as was seen in our patients. In all instances, IVS2+1G>A was in phase with the same haplotype 314;G;222;G. Three out of the four alleles were found in the same geographic focus (La Cañada, Zulia state); thus haplotype sharing and geographic aggregation strongly suggest a common remote ancestor and a founder effect as well, for these families in the country.

Mutation R48W had only one in-phase haplotype found in 2 unrelated patients, coming from different although contiguous geographic foci (in Lara and in Yaracuy states).The mutation is very infrequent worldwide (Choy *et al*, 2007), thus haplotype sharing might suggest a common and very remote origin for it in the northern occidental area of the country. R48W has a severe effect on the protein, as is suggested by its Polyphen score of 1.0.

Patients with Gaucher disease in Venezuela are geographically widespread, and their parents typically are not closely related, but 3 geographic foci were detected for 35% of the families. This suggests both remote kinship and a relatively high frequency of diverse mutant alleles,

beside a founder phenomenon. There was great genetic heterogeneity of mutations, with most of the patients being compound heterozygotes as is the usual finding everywhere, except among the Ashkenazim. However three mutations account for 72.5% of the chromosomes: N370S (42.5%), L444P (20%), and IVS2+1 G>A (10%). Thus, in any new patient, these mutations should be the first to be tested for in our populations, being they also the most common in European Caucasoids from diverse geographic regions, but not so in other ethnic groups.

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Table 1. Clinical and genetic epidemiological features of Gaucher disease index cases.

A: anemia; H: hepatomegaly; S: splenomegaly; T: thrombocytopenia; Bc: bone crisis; Hy; hypotonia; NA: not available data;

Patient/Sex		Age at diagnosis (years)	Clinical manifestations	Genotype[a]	In-phase haplotypes[b]	Geographic origin
1	♀	4	A ;S ; T	H311R N370S	314; G; ?; ? 318; G; ?; ?	HGO
2	♀	2	H; S; T; Hy	R48W ?	318; G; 222; A 322; G; 222; G	YSF
3	♀	2	A; H; S; T	R48W R120W	318; G; 222; A 326; G; 222, G	LSF
4	♂	9	A; H; T	N370S Rec*Nci*I	314; G; 222; A 314; G; 222; A	LSF
5	♂	33	NA	P245H N370S	314; G; 222; G 314; G; 222; G	HGO
6	♀	27	A; H, S; T; Bc	N370S [E326K+L444P]	314; G; 222; A 318; G; 222; G	LSF
7	♀	2	H; S; T	IVS2+1G>A N370S	314; G; 222; G 314; G; 222; G	HGO
8	♂	24	H, S	IVS2+1G>A N370S	314; G; 222; G 314; G; 222; G	ZSF
9	♀	8	A; H; S; T	IVS2+1G>A N370S	314; G; 222; G 314; G; 222; G	ZSF
10	♂	2	A; H; S; T	R433G L444P	318; G; 224; A 318; G; 224; G	HGO
11	♀	4	H; S; T	N370S L444P	314; G, 222; G 318; G; 222; G	HGO
12	♀	11	A; H; S	N370S ?	318; G; 224; G 318; G; 224; G	HGO
13	♀	8	S; T	IVS2+1G>A N370S	314; G; 222; G 314; G; 222; G	ZSF
14	♀	56	H; S	N370S ?	314; G; 222; A 314; G; 222; A	HGO
15	♀	50	H; S; Bc	Rec*Nci*I R496H	314; G; 222; A 318; G; 222; A	HGO
16	♂	9	A; H; S	N370S Rec*Nci*I	314; G; 222; A 318; G; 222; G	HGO
17	♀	21	H; S	W36X N370S	314; G; 222; G 314; G; 222; G	HGO
18	♂	44	S	N370S N370S	314; G; 222; G 314; G; 222; G	HGO
19	♀	13	H; S	N370S Rec*Nci*I	314; G; 222; A 314; G; 222; G	HGO
20	♂	18	H; S	N370S Rec*Nci*I	314; G, 222; A 318; G; 222; G	HGO

Rec*Nci*I: complex allele [L444P+A456P+V460V]; ?: unidentified genotype; HGO: heterogeneous geographic origins; ZSF: Zulia state focus, YSF: Yaracuy state focus, LSF: Lara state focus.

[a] Mutation nomenclature refers to the processed protein, not including the 39 residues of the signal peptide.

[b] Haplotype markers from left to right: ITG6.2; g.5470G>A (c.999+240G>A); 5GC3.2; g.4813G>A (c.762-180G>A).

Table 2. *GBA* mutations, in-phase haplotypes and its frequencies in patients and in a control sample.

GBA mutations	In-phase haplotypes	Haplotype frequencies (%)	
		Patients n= 40	Controls n=56
N370S Rec*Nci*I*	314; G; 222; A	22,5	14,3
R48W R496H	318; G; 222; A	7,5	26,8
L444P [E326K/L444P]* Rec*Nci*I*	318; G; 222; G	12,5	41,1
P245H W36X Rec*Nci*I* IVS2+1G>A N370S	314; G; 222; G	37,5	16,1
Unidentified	322; G; 222; G	2,5	1,8
N370S L444P	318; G; 224; G	7,5	0
R433G	318; G; 224; A	2,5	0
R120W	326; G; 222; G	2,5	0
N370S	318; G; (-); (-)	2,5	0
H311R	314; G; (-); (-)	2,5	0

Some haplotypes were found to be in phase with more than one mutation type, and some mutations had more than one in-phase haplotypes (N370S, L444P). Haplotype frequencies were different between patients and control individuals; the most frequent in controls (318;G;222;G) were not in patients chromosomes, suggesting that some mutations arose in a different genetic background. Some in-phase haplotypes were not found in the control sample.
n= number of chromosomes; (-) uninformative (n=2 chromosomes); * complex alleles; Rec*Nci*I: [L444P+A456P+V460V].
Haplotype markers from left to right: ITG6.2; g.5470G>A (c.999+240G>A); 5GC3.2; g.4813G>A (c.762-180G>A).