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RESEARCH REPORT

Biochemical and Molecular Chitotriosidase Profiles in Patients with Gaucher Disease Type 1 in Minas Gerais, Brazil: New Mutation in CHIT1 Gene

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Abstract Chitotriosidase (ChT) is a human chitinase secreted by activated macrophages and its activity is used in therapeutic monitoring of Gaucher disease (GD), the most common lysosomal storage disease. About 6% of the population is homozygous for a duplication of 24 bp in exon 11 of the CHIT1 gene (dup24), which is the main polymorphism that results in the absence of ChT. As ChT enzyme activity can be used as a biomarker in GD, it is important to know the CHIT1 genotype of each patient. In this study, ChT activity and CHIT1 genotype were evaluated in 33 GD type 1 patients under treatment in the state of Minas Gerais, Brazil, and compared to healthy controls. As expected, the enzyme activity was found to be higher in GD type 1 patients than in healthy subjects. Four patients had no ChT activity. Their genotype revealed three patients (9%) homozygous for dup24 allele and one patient with two polymorphisms in exon 11: G354R and a 4 bp deletion at the exon-intron 11 boundary (g.16993_16996delGAGT), the later described for the first time in literature. Two other patients with lower ChT activity presented a polymorphism in exon 4 (c.304G>A, p.G102S), without dup24 allele. In conclusion, this study demonstrated that ChT activity can be used for therapeutic monitoring in 82% of GD patients of the state of Minas Gerais, Brazil.

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Introduction

Gaucher disease (GD; MIM 230800), the most prevalent of the sphingolipid storage disorders, is caused by a genetic defect that leads to glucocerebrosidase (β-glucosidade) deficiency (EC 3.2.1.45) and progressive accumulation of its substrate, glucocerebroside, in cells of monocyte/macrophage origin (Brady et al. 1965; Beutler and Grabowski 1995). Most patients present progressive visceral enlargement and gradual replacement of the bone marrow with lipid-laden macrophages, hematological abnormalities, and structural skeletal changes. Progressive neurologic deterioration develops in a minority (Beutler and Grabowski 1995).

Patients with GD type 1 are commonly treated by enzyme replacement therapy (ERT) with imiglucerase, only produced by Genzyme Corporation (Cambridge, MA, UK), a high-cost treatment provided by the Brazilian Ministry of Health. The parameter most frequently employed to monitor effectiveness of this therapeutic intervention is the activity of chitotriosidase (ChT) (Cox et al. 2000; Hollak et al. 1994, 2001; Mistry and Abrahamov 1997), a chitinase encoded by the chitotriosidase gene (CHIT1; MIM 600031). The CHIT1 gene is currently known to possess 13 exons with sizes ranging from 30 to 1055 bp (NG_012867.1, National Center for Biotechnology Information, 2012; http://www.ncbi.nlm.nih.gov/gene/1118).

The enzyme is synthesized by activated macrophages and is usually highly increased in serum from GD type 1 patients (Froissart 2006; Hollak et al. 1994). However, monitoring therapeutic response by measuring plasma ChT activity has a pitfall: the complete absence of enzymatic activity in approximately 6% of individuals (Boot et al. 1998). This observation can be explained by the homozygozity for a duplication of 24 bp in exon 11 of the CHIT1 gene (dup24, rs3831317) preventing formation of active enzyme (Boot et al. 1998). However, dup24, the main mutation described, is not the only one with implications for diagnosis and therapeutic monitoring of patients. Some polymorphisms, such as G102S (c.304G>A, p.G102S, rs2297950), G354R (c.1060G>A, p.G354R, rs9943208), and A442V (c.1325C>T, p.A442V, rs1065761) have been associated with reduced ChT activity (Bussink et al. 2009; Grace et al. 2007; Lee et al. 2007).

The relative scarceness of data regarding the incidence of GD in Brazil, the evidence that, as a group, Brazilian patients may have a more aggressive form of the disease (Sobreira et al. 2007), and the high cost of ERT to the public health budget prompted us to study the biochemical and molecular ChT profiles in an effort to assess treatment efficacy. Indeed, in 2007, about 500 patients with GD type 1 were treated in Brazil with 177,280 bottles of ERT being purchased at a cost of over US$ 125 million (Souza et al. 2010). The effective dose of imiglucerase is individually adjusted according to the clinical status and treatment response of the patient. The dose ranges from 15 to 60 U/kg and is supplied every 2 weeks (Martins et al. 2009). For this purpose we correlated the measure of plasma ChT activity with CHIT1 genotypes of GD type 1 patients from the Hospital das Clinicas of the Universidade Federal de Minas Gerais (HC-UFMG), and compared with healthy controls. Ultimately we reasoned that such studies could be valuable for the monitoring of ERT effectiveness and help physicians to devise the best treatment protocol strategies which would represent a more adequate use of treatment resources and improvement of therapy results for patients. We found that 9% of the patients analyzed (P1, P2, and P3) presented null or low ChT activity levels in spite of homozygous or heterozygous for the dup24 wild type allele.

Materials and Methods

Samples

Seventeen female and 16 male GD type 1 patients under treatment (32 in ERT with imiglucerase and one in substrate reduction therapy – SRT) at the reference center in HC-UFMG participated of this study. The median age of the patients was 27 years (range: 5–62 years) and the median treatment time was 11 years (range: 1–14 years). All patients had diagnosis confirmed by measurement of glucocerebrosidase activity in leukocytes (Beutler and Kuhl 1970) or cultured fibroblasts (Beutler et al. 1971). The control group consisted of 33 healthy volunteers of which 18 were females and 15 were males. The median age of the control group was 24 years (range: 8–52 years).

Ethics

The study was approved by the Ethics in Research Committee of Universidade Federal de Minas Gerais, under the numbers ETIC 625/07 and ETIC 0414.0.203.000-09, and informed consent was obtained from all participants.

CHIT1 Genotyping

Samples of 4 mL of peripheral blood were collected from GD type 1 patients in tubes containing EDTA and immediately subjected to DNA extraction according to the method described by Miller and coworkers (1988). DNA samples from 33 healthy controls were collected using buccal swabs and extracted with the BuccalAmp™ DNA Extraction Kit (Epicentre, Madison, WI, USA). The DNA samples were stored at −20 °C until molecular analysis.

Genotyping for dup24 in CHIT1 gene was performed by PCR using specific primers (ChTF: AGCTATCTGAAG-CAGAAG and ChTR: GGAGAAGCCGGCAAAGTC) as previously described (Boot et al. 1998). The 20 μL reaction contained 1x PCR Buffer (Sigma-Aldrich Co., St. Louis, MO, USA), 0.2 mM dNTPs, 0.2 mM of each primer, 1.25 mM $MgCl_2$, 0.5 U Taq polymerase (Sigma-Aldrich Co., St. Louis, MO, USA), and 50 ng of DNA template. The DNA was denatured at 95 °C for 2 min, and amplification was performed by 35 cycles at 95 °C for 20 s, 56 °C for 20 s and 72 °C for 30 s. The amplified fragments were

Table 1 Primers used for analysis of the CHIT1 polymorphisms

Polymorphisms	Primers (5'–3')	Fragment Size	Annealing temperature	Reference
G102S	F: ACATAGGCACTTTCACACGTC R: AACAGCCTGGAGCAAAGCTC	380 bp	56 °C	Lee et al. (2007)
G354R*	F: GAATCTACAGCCACTCACAGG R: CAGGTAAGAGAGGAACAAGG	551 bp	56 °C	–
A442V*	F: GGGGAGCCTTGGTTGAATCT R: TGAGAGCAGAAAGCCTGGATA	423 bp	56 °C	–

* The primers for analysis of the G354R and A442V polymorphisms were based on CHIT1 genomic sequence from NCBI database (NG_012867.1)

separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. Genotypes were determined by the presence or absence of wild type (75 bp) and/or mutant (99 bp) bands.

The three patients (P1, P2, and P3) who presented null or low levels of ChT activity in spite of being homozygous or heterozygous for the dup24 wild type allele were screened for three polymorphisms in CHIT1 that correlate with reduced ChT activity: G102S, G354R, and A442V (Bussink et al. 2009; Grace et al. 2007; Lee et al. 2007). PCR amplification of the regions containing the polymorphisms G102S (exon 4), G354R (exon 11), and A442V (exon 13) was performed using specific primers detailed in Table 1. The 40 μL reaction contained 1x PCR Buffer (Sigma-Aldrich Co., St. Louis, MO, USA), 1.5 mM dNTPs, 0.2 mM of each primer, 2.5 mM $MgCl_2$, 1 U Taq polymerase (Sigma-Aldrich Co., St. Louis, MO, USA), and 100 ng of DNA template. The DNA was denatured at 95 °C for 2 min, and amplification was performed by 35 cycles at 95 °C for 20 s, 56 °C for 20 s, and 72 °C for 30 s. The amplified fragments were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

Fragments were purified from the agarose gel using the Promega Wizard® SV Gel and PCR Clean-up System kit (Promega Corporation, Madison, WI, USA) as recommended by the manufacturer. The purified samples were sequenced (forward and reverse direction) in the ABI 3130 Genetic Analyzer (Applied Biosystems Inc, Foster City, CA, USA), using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc, Foster City, CA, USA) as recommended by the manufacturer. Sequences were analyzed and aligned to the reference sequence of CHIT1 gene NG_012867.1 using the CodonCode Aligner software (http://www.codoncode.com/aligner/).

To confirm heterozygosity for the G102S polymorphism in patient P2, the fragment containing the possible alteration was cloned. Cloning was performed on PCR®II-TOPO® plasmid using the TOPO TA Cloning Kit (Invitrogen™ Life Technologies, Carlsbad, CA, USA) as recommended by the manufacturer.

Plasma ChT Activity

Plasma was obtained by centrifugation from peripheral venous blood collected in EDTA and stored at –20 °C until enzymatic analysis at the Laboratory of Inborn Errors of Metabolism of HC-UFMG. The measurement of the ChT activity was performed as previously described by Hollak and coworkers (1994). Briefly, 5 μL of diluted plasma was incubated with 200 μL of a solution containing 0.022 mM of the artificial substrate 4-methylumbelliferyl-β-D-N-N′-N″-triacetylchitotrioside (Sigma-Aldrich Co., St. Louis, MO, USA) in citrate-phosphate buffer (0.1/0.2 M) (Sigma-Aldrich Co., St. Louis, MO, USA), pH 5.2, for 15 min at 37 °C. The reaction was interrupted by adding 2 mL of 0.3 M glycine buffer (Sigma-Aldrich Co., St. Louis, MO, USA), pH 10.6. The fluorescent 4-MU was measured with a spectrofluorometer Hitachi F-2500 (Hitachi High-Technologies Corporation, Tokyo, Japan), excitation (EX) at 366 nm and emission (EM) at 446 nm. The slit width was 5 nm, both EM and EX. The coefficient of variation (CV) between duplicates was 5%, which makes the test highly reproducible (Canudas et al. 2001; Comabella et al. 2009; Vellodi et al. 2005). ChT activity was expressed in nanomols of hydrolyzed substrate per hour per mL (nmol/h/mL).

Statistical Analysis

Analyses were performed using the statistical software package SPSS 14.0. Results are presented as median and range (minimum and maximum). Four GD type 1 patients and three healthy controls showed no detectable ChT activity and therefore were excluded from further statistical analysis (Canudas et al. 2001). The nonparametric Mann–Whitney test was used in order to evaluate differences in ChT activity between GD type 1 patients and controls, and also among patients and control individuals grouped by genotype. The same test was used to verify the effect of gender on enzyme activity. The relations between ChT activity and age were tested by the Spearman correlation. Chi-square test was used to evaluate differences in

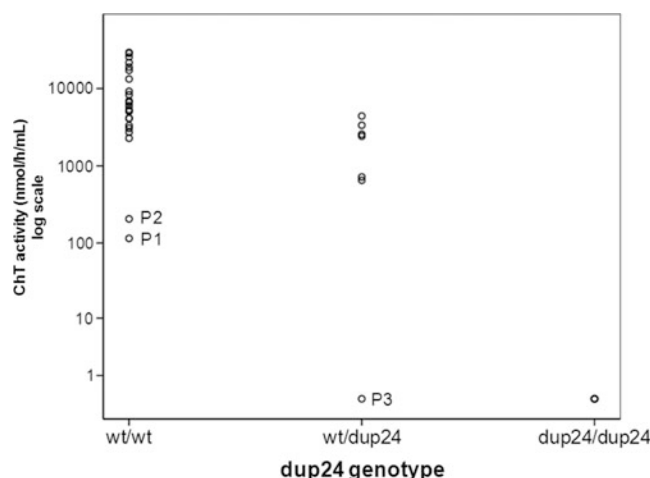

Fig. 1 Levels of ChT activity in 33 GD type 1 patients. Circles represent the ChT activity in each GD type 1 patient evaluated conform dup24 genotype. The enzyme activity was undetectable in patients with dup24/dup24 genotype ($n = 3$) and in patient P3 and reduced in patients with wt/dup24 genotype ($n = 7$). Patients P1, P2, and P3 are highlighted on the graph

frequency of dup24 genotype between genders in GD type 1 patients. In all tests, the level of significance was set at $p < 0.05$.

Results

Of the 33 GD type 1 patients, 23 (70%), seven (21%), and three (9%) were, respectively, wild type (wt/wt), heterozygous (wt/dup24), and homozygous (dup24/dup24) mutants for the allele dup24 (Fig. 1). Among healthy controls, 15 (45.5%), 15 (45.5%), and three (9%) showed, respectively, wt/wt, wt/dup24, and dup24/dup24 genotypes. The frequency of the wt and dup24 alleles among patients was 0.803 and 0.197, respectively, and 0.682 and 0.318 in the control group. The genotype distribution was in Hardy-Weinberg equilibrium.

As expected, plasma ChT activity was 176 times higher in 29 treated patients with GD type 1 (median: 5092, range: 115–29284 nmol/h/mL) than in control group (median: 29, range: 7–68 nmol/h/mL; $p < 0.0001$) (Table 2). Patients with wt/wt genotype (median: 6011, range: 115–29284 nmol/h/mL) showed ChT activity 194 times higher than healthy controls with the same genotype (median: 31, range: 17–68 nmol/h/mL; $p < 0.0001$). Patients with the genotype wt/dup24 (median: 2496 nmol/h/mL, range: 652–4417 nmol/h/mL) showed ChT activity 119 times higher than healthy wt/dup24 controls (median: 21, range: 7–52 nmol/h/mL; $p < 0.0001$) (Table 2). GD type 1 patients and controls with the genotype dup24/dup24 presented no ChT activity. Considering GD type 1 patients with different genotypes, the ChT activity was about twice lower in patients with the genotype wt/dup24 when compared to patients with the wt/wt genotype ($p < 0.05$).

No correlation was observed between ChT activity and age in both populations ($p > 0.05$). No relationship was noted between gender and ChT activity ($p > 0.05$) or gender and distribution of dup24 genotype ($p > 0.05$) in GD type 1 patients.

Two homozygous (P1 and P2) and one heterozygous (P3) patients for the dup24 wild type allele showed lower ChT activity levels than the average reported in their group (Fig. 1). Consequently, they were screened for three polymorphisms that have been shown to reduce ChT enzyme activity. We found that patient P1 (ChT activity of 115 nmol/h/mL) was homozygous for the polymorphism G102S, which is a guanine to adenine transition at position 304 of the cDNA (c.304G>A). Patient P2 (ChT activity of 206 nmol/h/mL) was heterozygous for the same polymorphism. Cloning confirmed the heterozygosity in this patient. Patient P3 (null ChT activity) presented two alterations in exon 11. This patient was found to be heterozygous for the polymorphism G354R, which is a guanine to adenine transition at position 1060 of the cDNA (c.1060G>A) and for a 4-base deletion in exon-intron 11 boundary (g.16993_16996delGAGT; rs143439055) (Fig. 2). This is the first report on this deletion to date.

None of the patients evaluated presented the polymorphism A442V, which is a cytosine to thymine transition at the position 1325 of the cDNA (c.1325C>T).

Discussion

To achieve a better monitoring of GD type 1 patients and correlate ChT activity with treatment efficacy we measured the enzyme levels and correlated them with the CHIT1 genotype of each of the 33 GD type 1 patients under treatment.

Our results showed that the ChT activity was higher in most GD type 1 patients analyzed than in the healthy controls, which is in accordance with previous studies (Hollak et al. 1994; Ries et al. 2006; Wajner et al. 2004, 2007). Wajner and colleagues (2004, 2007) reported that the ChT activity was around 600-fold greater in untreated GD patients of Brazilian origin than in healthy individuals. In the present study, GD type 1 patients also showed an elevation in the levels of enzyme activity; however, it was only 176 times higher. The divergence may be explained by the fact that all patients in our study were under treatment, whereas patients reported by Wajner and colleagues were receiving no treatment. We observed that ChT activity in GD type 1 patients homozygous wild type was approximately twice higher than in heterozygous patients. This result is in accordance with Schoonhoven and co-authors (2007), and

Table 2 Dup24 genotype and ChT activity in GD type 1 patients and controls

	N° of subjects	Genotype (%)	ChT activity (nmols/h/mL) Median (minimum–maximum)
GD type 1	33	100	5092 (115–29284)
wt/wt	23	70	6011 (115–29284)
wt/dup24	7	21	2496 (652–4417)
dup24/dup24	3	9	0
Control group	33	100	29 (7–68)
wt/wt	15	45.5	31 (17–68)
wt/dup24	15	45.5	21 (7–52)
dup24/dup24	3	9	0

Fig. 2 4-base deletion (g.16993_16996delGAGT) at the exon-intron 11 boundary in patient P3. This deletion involves the last two bases of exon 11 (GA) and the first two positions of intron 11 (GT). The nomenclature g.16993_16996delGAGT was based on CHIT1 genome sequence from NBCI (NG_012867.1)

can be explained by the presence of the null allele dup24, which does not produce active enzyme (Boot et al. 1998).

The causes for this activity increase are not yet established. Some studies suggest that in GD type 1 patients the accumulation of the immunogenic components in macrophages, such as ceramide and sphingolipids, causes cellular activation and consequently ChT secretion, which may mediate the immune response involved (Ballou et al. 1996; van Eijk et al. 2005). Increased ChT activity was also recorded in several other diseases, such as Niemann-Pick (Brinkman et al. 2005), GM1 gangliosidosis (Malaguarnera et al. 2003), β-thalassemia (Barone et al. 1999), sarcoidosis (Boot et al. 2010), malaria (Barone et al. 2003), atherosclerosis (Artieda et al. 2003; Boot et al. 1999), and fungal and bacterial infections (Iyer et al. 2009; Labadaridis et al. 1998). The role of ChT enzyme is unclear, but a possible role in defense against chitin-containing pathogens and host immune response has been suggested (Choi et al. 2001; Di Luca et al. 2007; Di Rosa et al. 2005; Gordon-Thomson et al. 2009; Malaguarnera et al. 2005; van Eijk et al. 2005).

It is estimated that approximately 6% and 33–35% of the general population is homozygous and heterozygous for the mutation dup24, respectively (Boot et al. 1998). In our study, we found that 9% and 21% of GD type 1 patients present the genotypes dup24/dup24 and wt/dup24, respectively. Among healthy controls, 9% were dup24/dup24 and 45.5% were wt/dup24. The deviations observed can be attributed to the not large enough number of evaluated samples, and also to selection bias, since among GD type 1 patients there were six families, and twelve related individuals. The Hardy-Weinberg test revealed that the frequency of dup24 genotypes in GD type 1 patients and healthy controls is in equilibrium. However, the p value observed for the group of GD type 1 patients was $p = 0.06$, which is very close to the level of significance cut-off. This may also be due to the number of patients genotyped and/or selection bias.

Genotyping of polymorphisms in the CHIT1 gene for three patients (P1, P2, and P3) who presented divergent correlation between dup24 genotype and ChT activity revealed that patients P1 and P2 were, respectively, homozygous and heterozygous for G102S polymorphism. Grace and coworkers (2007) showed, using 4-MU-chitotrioside as substrate, that recombinant expressed G102S ChT had only 23% residual activity in relation to the wild type ChT. On the other hand, Lee and colleagues (2007) analyzing GD type 1 patients noted that this polymorphism was not associated with reduced enzyme activity. Bussink and co-authors (2009) reported that G102S affects differentially the ChT activity depending on the substrate used for enzyme measurement: when the substrate 4-MU-chitotrioside was used, the mutated ChT containing Ser102 showed reduced catalytic efficiency; whereas normal catalytic efficiency of the enzyme containing Ser102 was observed when the substrate 4-MU-deoxychitobioside (Aguilera et al. 2003) was used. In the present study, we used the substrate 4-MU-chitotrioside, and this may explain the low enzyme activity observed. Furthermore, the mentioned authors reported a considerable overlap of results and a relatively high frequency of the G102S allele in different ethnic groups. Thus, we cannot exclude the possibility that GD type 1 patients that were not genotyped do not present this polymorphism.

The patient P3 showed more complex changes in CHIT1 gene. Besides presenting the wt/dup24 genotype, this patient is heterozygous for the G354R polymorphism in exon 11, which has been previously associated with reduced ChT activity (Grace et al. 2007; Lee et al. 2007). Interestingly, this patient presented a 4-base deletion at the exon-intron 11 boundary (g.16993_16996delGAGT), first reported in this study. This deletion involves the last two bases of exon 11 and the first two positions of intron 11, altering the 5’ donor splice-site. A similar genotype was described by Grace and colleagues (2007), whose patient with GD type 1 showed three alterations, called “complex E/I-10 allele”: a G354R polymorphism, a silent base change (L385L) in exon 11, and a 4-base deletion in intron 11. The mutation described in the present study changes the 5’

donor splice-site, leading to a splice error which may underlie the absence of ChT activity observed in patient P3. This mutation may produce a truncated ChT enzyme, but future studies will be needed to confirm this hypothesis.

In conclusion, this study characterized the ChT activity in 33 GD type 1 patients from Minas Gerais state under ERT and SRT treatment and described a novel mutation in the CHIT1 gene with important implications for diagnosis and therapeutic monitoring of patients. We found that ChT activity cannot be used as biomarker in 18% of GD patients evaluated, since these patients had null (four patients) or low (P1 and P2) ChT activity. Such knowledge can improve the planning of treatment aiming at improving the response of GD type 1 patients to ERT and SRT.

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Synopsis

Biochemical and molecular chitotriosidase profiles in Brazilian patients of Minas Gerais state, affected by Gaucher disease type 1, including the discovery of a new mutation in CHIT1 gene, are reported.

Conflict of Interest

None declared

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