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Clinical and molecular characteristics of patients with Gaucher disease in Southern China

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ABSTRACT

Gaucher disease (GD) is a common lysosomal storage disorder caused by the deficiency of acid β-glucosidase, due to mutations in the *GBA* gene. To explore the clinical and molecular characteristics of GD patients from Southern China, *GBA* gene was analyzed by nest PCR and direct Sanger-sequencing. Novel missense mutations were transiently transfected in COS-7 cells by plasmid system for functional verification. Among the 22 GD patients, 19 patients were classified as type 1 and three as type 2. Over 60% of the type 1 patient had onset before 2 years old and about 42% of them died before 3 years old. Six type 1 patients with L444P homozygous genotype, presented with early onset and severe hepatosplenomegaly. Four novel mutations Y22C, F109 L, L149F and c.983_990delCCCACTGG were identified. The GBA activities in vitro of novel mutants Y22C, F109 L and L149F were 20.2%, 6.9% and 6.5% of the wild-type, respectively. L444P mutation accounted for 47.7% of the mutant alleles. Our results revealed that type 1 GD tends to present with a severe phenotype among southern Chinese. L444P was the most prevalent mutation and L444P homozygote genotype was associated with severe type 1 GD. Three novel missense mutations identified were pathogenic.

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1. Introduction

Gaucher disease (GD, MIM 230800) is a common lysosomal storage disorder. It is caused by the deficiency of the lysosomal enzyme glucocerebrosidase (GBA, EC 3.2.1.45), which catalyzes the breakdown of the glucosylceramide to glucose and ceramide. The disease occurs at a frequency of 1/50,000 to 1/100,000 in the general population [1]. Three main clinical forms of GD have been described: Type 1 non-neuronopathic, Type 2 acute neuronopathic, and Type 3 subacute neuronopathic. Type 1 GD is the most common form accounted for 95% in Jewish and non-Jewish Caucasian cases of GD, but neuronopathic form (Type 2 and Type 3 GD) comprises over half of GD patients in Asian (61.1% in Korean, 56.5% in Japanese and 42.0–57.9% in Chinese) [1–3].

Gaucher disease is mainly caused by the mutations in the glucocerebrosidase gene (*GBA*, MIM No.606463). The *GBA* gene, spans ~7.5 kb of genomic DNA, is located on chromosome 1q21 and composed of 11 exons [4]. A highly homologous ~5.5 kb pseudogene (*GBAP*, MIM No. 606463), which shares 96% exonic sequence homology, is located 16 kb downstream from the functional gene [5]. To date, at least 437 different mutations causing GD have been identified (HGMD Professional 2016.1). The mutation spectrum of *GBA* in Asians is quite different from that in Ashkenazi Jewish patients and non-Jewish patients, where N370S, 84insG, L444P and IVS2 + 1G > A mutations account for about 97% and 75% of the mutant alleles, respectively [6]. In Asians, L444P, F213I and Rec*Nci*I mutations are the most common mutants [3,7–9]. There are few clear genotype/phenotype correlations in GD: N370S and N188S mutations are related with Type 1 GD [9,10]; L444P/Rec*Nci*I phenotype is associated with Type 2 GD [8,11].

Therapeutic approaches for GD include enzyme replacement therapy (ERT), substrate reduction therapy (SRT), pharmacological chaperone therapy (PCT) and gene therapy. Although ERT and SRT can improve the symptoms significantly, they are not effective in Type 2 and 3 GD. PCT is a promising alternative therapeutic approach for its ability to penetrate the blood–brain barrier to treat the neuronopathic forms. Recently, ambroxol was described as an effective chaperone on fibroblasts with L444P/L444P and L444P/Rec*Nci*I genotype [12]. In addition, ambroxol was confirmed to be effective in the GD patients with N188S, F213I or Rec*Nci*I heterozygote [13].

We intended to explore the clinical and molecular characteristics of *GBA* mutations and to further analyze the relationship between genotype and phenotype of GD in southern Chinese, which helps us to predict the prognosis of patients and to offer optimal therapeutic approaches.

Abbreviations: GD, Gaucher disease; GBA, acid β-glucosidase; cDNA, complementary deoxyribonucleic acid; PCR, polymerase chain reaction; EGFP, enhance green fluorescent protein; WT, wild-type.

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2. Materials and methods

2.1. Patients

Twenty-two unrelated GD patients from Southern China (Guangdong, Hunan, Jiangxi and Guizhou Province) were enrolled in this study. Fifteen were male and seven were female. The age of diagnosis ranged from 5 months to 69 years old (63% of them <4 years old). The diagnosis of GD was based on clinical findings and confirmed by the deficiency of GBA activity in peripheral leukocytes measured by Guangzhou Women and Children's Medical Center from February 2010 to March 2015. Follow-up was conducted for all patients for 1.4–6.5 years (at a mean time 3.5 years) through clinical or telephone counseling. Samples from 50 unrelated healthy Chinese volunteers were taken as controls. Informed consent was obtained from adult parents and parents of children. This study was approved by the Ethics Committee of Guangzhou Women and Children's Medical Center.

2.2. GBA gene sequencing

Genomic DNA was extracted from peripheral blood samples. Nested PCR approach was used to selectively amplify the *GBA* gene but not the pseudogene sequence [10]. Primers (Table S1) were designed by Primer 5 software (Biosoft International, Palo Alto, USA). The coding and splicing regions of the *GBA* gene were amplified with Ex Taq DNA polymerase (TaKaRa Bio Inc., Otsu, Japan). The PCR products were sequenced by an ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA) and the sequencing chromatograms were analyzed by comparison with the corresponding wild-type *GBA* sequence (GenBank: NG_009783.1) using Sequencher software DNAMAN (Lynnon Biosoft, Inc., Quebec, Canada). Once variants were identified, the PCR and sequencing on the corresponding exons were repeated at least twice to verify reliability. Pathogenicity of novel missense variants was analyzed using Sorting Intolerant From Tolerant (SIFT) (http://sift.jcvi.org/) and Polymorphism Phenotyping v2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/pph2/index/shtml). In addition, the novel variants identified were examined in 50 normal controls.

2.3. Plasmid construct

The wild-type *GBA* cDNA (GenBank: NM_000157.2) was linked to the enhanced green fluorescent protein (EGFP) reporter gene and cloned into the pcDNA3.1 plasmid (Invitrogen, Carlsbad, California). Seven mutations of *GBA* gene, including three novel missense mutations (Y22C, F109 L and L149F) and four previous reported mutations (L444P, N188S, L96P and L246I), were introduced in the wild-type cDNA fused to EGFP by a Quikchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and confirmed by sequencing. L444P, L246I and N188S mutations were performed as positive controls. L96P mutation was reported previously without functional. The primers used for site-directed mutagenesis were listed in Table S2.

2.4. Cell culture and transfection

COS-7 cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NJ, USA) supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NJ, USA), 100 U/mL penicillin and 100 U/mL streptomycin (Hyclone, Logan, UT, USA) at 37 °C in a humidified atmosphere enriched with 5% CO_2. COS-7 cells were transfected by pcDNA3.1-EGFP vector, *GBA* wild-type and seven mutants, respectively, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 72 h transfection, cells were harvested for GBA enzyme assays and Western blotting. All transfections were performed in duplicate in three individual experiments.

2.5. GBA activities

The harvested COS-7 Cells were sonicated in ddH_2O and then protein concentrations were determined using Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA). GBA activities were measured using substrate 4-methylumbelliferyl-β-D-glucoside (4MU-Glu, Sigma, St. Louis, MO, USA) [14]. Briefly, 10 μL of the protein homogenates (15 μg) was incubated with 20 μL of 15 mM 4MU-Glu containing 5.76 mM taurodeoxycholic acid (Sigma, St. Louis, MO, USA) at 37 °C for 2 h. And 200 μL of 0.2 M glycine buffer (pH 10.5) was then added and the fluorescence was measured by fluorimeter (BioTek Flx800, USA) (excitation at 355 nm, emission at 460 nm). The enzyme activities of mutants were expressed as the percentages of wild-type enzyme activity. Acid β-galactosidase was detected as a reference enzyme.

2.6. Western blotting

The transfected cells were lysed by RIPA Lysis Buffer (Beyotime, Beijing, China) containing 1% PMSF (Beyotime, Beijing, China). Protein (30 μg) was loaded for electrophoresis on 8% SDS-PAGE gels and transferred to PVDF membranes. The membranes were then blocked with 5% non-fat milk in TBS, containing 0.1% Tween 20 for 1.5 h at room temperature, and then incubated with antibodies against GBA (1:1000) (Abcam Inc., Cambridge, MA, USA) at 4 °C overnight. Finally, membranes were incubated with a secondary goat *anti*-mouse IgG (1:5000) (Boster Bio Inc., Wuhan, China) for 2 h at room temperature. The signals were detected using the ECL western blotting detection reagent (Pierce, Rockford, IL, USA).

2.7. Structural analysis of mutations

The *GBA* novel mutations were analyzed using the X-ray structure (PDB-code: 1OGS) and amino-acid substitutions were carried over using the PyMOL (Delano Scientific LLC, San Carlos, CA, USA) and Discovery Studio (Accelrys Inc., San Diego, CA, USA). Figures were drawn using Photoshop CS3 (Adobe Systems, San Jose, CA, USA).

2.8. Statistical analysis

The difference between the wild-type enzyme activities and the individual mutant enzyme activities was analyzed by GraphPad Prism 5 (GraphPad Software Inc., CA, USA) using a Student's *t*-test. *P* values of ≤0.05 were considered statistically significant.

3. Results

3.1. Clinical features

As shown in Table 1, the GBA activities in leukocytes of GD patients were significantly reduced compared to normals (2.05 ± 1.49 nmol/mg/h vs. normal reference range 10–25 nmol/mg/h). Among the 22 GD patients, 19 patients were classified as Type 1 and three as Type 2. Over 63% (12/19) of the type 1 patients had onset at <2 years old and presented severe hepatosplenomegaly and hematological complications (anemia, thrombocytopenia, epistaxis) and about 42% (5/12) of them died before 3 years old. The three Type 2 patients had onset before 6 months and quickly progressed to present opisthotonus, dystonia, ophthalmoplegia, psychomotor retardation and severe hepatosplenomegaly. All of them died before 2 years old. Except for two patients (10th and 15th) treated with bone marrow transplantation and enzyme replacement therapy, respectively, the patients did not receive any specific therapy.

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