Original Article

A Novel Functional Missense Mutation p.T219A in Type 1 Gaucher's Disease

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Abstract

Background: Gaucher's disease (GD) is an autosomal recessive disorder caused by a deficiency of acid β-glucosidase (glucocerebrosidase [GBA]) that results in the accumulation of glucocerebroside within macrophages. Many mutations have been reported to be associated with this disorder. This study aimed to discover more mutations and provide data for the genetic pattern of the gene, which will help the development of quick and accurate genetic diagnostic tools for this disease.

Methods: Genomic DNA was obtained from peripheral blood leukocytes of the patient and Sanger sequencing is used to sequence *GBA* gene. Sequence alignments of mammalian β-GBA (GCase) and three-dimensional protein structure prediction of the mutation were made. A construct of this mutant and its compound heterozygous counterpart were used to measure GCase *in vitro*.

Results: GCase is relatively conserved at p.T219A. This novel mutation differs from its wild-type in structure. Moreover, it also causes a reduction in GCase enzyme activity.

Conclusion: This novel mutation (c.655A>G, p.T219A) is a pathogenic missense mutation, which contributes to GD.

Key words: Gaucher's Disease; GCase; Genetics; Novel Missense Mutation

Introduction

Lysosomal storage diseases comprise a group of inherited metabolic diseases caused by genetic mutations that lead to enzymatic abnormalities in the lysosome organelle. The corresponding substrates are then accumulated.[1] The overall incidence rate of these diseases was approximately 1:5000.[2] Gaucher's disease (GD) includes several clinical subtypes with a continuum of clinical findings from a perinatal lethal disorder to an asymptomatic type. The clinical diagnosis of GD currently relies on demonstration of a deficient lysosomal enzyme acid β-glucosidase (glucocerebrosidase [GBA]) which is responsible for the hydrolysis of glucocerebroside (glucosylceramide) into glucose and ceramide.[3,4] *GBA* gene is the only gene in which mutations are known to cause the GD. Mutations of the gene encoding β-GBA (GCase) cause varying degrees of decreased GCase activity. The accumulated substrates lead to organ damage of diseased individuals, particularly in the liver, spleen, bone marrow, and brain.[5,6] Three major clinical forms of GD are mostly distinguished by clinical manifestations, age onset, and rate of disease progression.[7] GD Type 1 is characterized by the presence of clinical or radiographic evidence of bone symptoms, hepatosplenomegaly, anemia, and thrombocytopenia, and the absence of primary central nervous systemic symptoms.

Mature acid ß-GBA is a 59 kD, 497 amino acid membrane glycoprotein encoded by the *GBA* gene (GenBank accession #J03059). *GBA* gene is localized on chromosome 1q21, spans 7.6 kb and is composed of 11 exons and ten introns.[8]

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To date, more than 420 mutations have been reported in *GBA* gene, including point mutations (missense and nonsense), deletions and insertions, splice junction mutations, frame shifts alterations, and recombinant alleles. It is not enough of these known mutations for the study of GD, and much more novel mutations remain to be discovered. In Jewish descent, N370S (c.1226A>G) was found to be the most common mutation.[9] Worldwide, the incidence of GD varies by ethnicity. This study aimed to discover more mutations and provide data for the genetic pattern of *GBA* gene, which will help the development of quick and accurate genetic diagnostic tools for this disease.

Methods

Patient and clinical data

All procedures were approved by the Institutional Review Board of Shanghai Children's Hospital, China. Experiments were carried out only after written informed consent had been obtained from the parents of subjects younger than 18 years.

A 5-year-old boy was full-term delivered by his mother at the age of 36-year-old with a procedure of cesarean. He had no siblings in his family. His parents were Chinese of Han origin and were nonconsanguineous. When he was 3-year-old, he suffered from pneumonia. Ultrasound scanning revealed that he had an enlarged liver compared to the normal child. However, his liver function was normal at that time. No dermal ecchymosis or superficial lymph node enlargement was ever detected. No cyanotic phenomenon was ever observed. He never had bone pain in the history. He came to the clinic with a claim of development delay by his mother. He had a rough breathing without obvious lung symptoms. Physical examination identified that liver was located about 3 cm under the rib cage, and spleen was located about 15 cm under the left side of the rib cage. No touch pain was observed. X-ray showed no abnormal periosteal elevation or other signs except that slight endosteal scalloping enlargement was noted. Abdominal computed tomography scanning showed obvious splenomegaly. His blood test showed that his white blood cell was 7.1×10^9/L, platelet 74×10^9/L, Hb 112 g/L, reticulocyte 1.7%. No obvious abnormality was observed by head magnetic resonance imaging. Bone marrow examination revealed the presence of lipid-engorged macrophages, typical Gaucher cells appeared with "fibrillary crumpled silk" in the cytoplasm and eccentrically placed nucleus. To confirm the diagnosis of GD, we performed the assay of GBA enzyme activity in Xinhua Hospital, China. The enzyme activity in peripheral blood leukocytes was 1.94 nmol·h^{-1}·mg^{-1}, about 3.5–29.5% of normal activity (6.56–55.10 nmol·h^{-1}·mg^{-1}).

Molecular analysis

Genomic DNA was obtained from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. *GBA* gene is localized on chromosome 1q21, its pseudogene *GBAP1* is 16 kb downstream of *GBA*. The similarity of them was 55% (9750 bp/17,575 bp) with a gap of 7025 bp. We designed long polymerase chain reaction (PCR) primers shown in Table 1 to distinguish *GBA* gene from *GBAP1*. PCR was performed as follows: DNA was predenatured for 5 min at 94°C, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s and elongation at 72°C for 2 min, and another 25 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and elongation at 72°C for 3 min, finally 72°C for 2 min. Then, designed primers are shown in Table 2 for each exon to sequence long PCR segments.

Sequencing reactions were performed with Sanger sequencing chemistry using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA) on an ABI 3130 automated sequencer (Applied Biosystems Inc., Foster City, CA, USA). Sequencing reaction conditions were performed as follows: predenatured for 1 min at 96°C, followed by 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and elongation at 60°C for 4 min. Data from sequencing were analyzed by Sequencher Demo 3.0 (Gene Codes Corp, USA) and Mutation Surveyor Demo version 4.0 (SoftGenetics LLC, USA) with the reference sequences from NCBI (NM_001005741.2 for GBA).

Phylogenetic analysis and structural predictions

Phylogenetic analysis was performed by Clustal × 1.83 programs to compare the human wild-type GCase protein sequence (NP_001005742.1) with orthologs from rat, mouse, pig, sheep, and cow. All parameters were default values of the software. We used SWISS-MODEL (http://swissmodel.expasy.org) to predict possible structural changes caused by T219A mutation and analyzed with PyMOL (Schrödinger, Japan). The further analysis of the stability of GCase was based on the prediction of the hydrophobic property of the protein by ProtScale (http://web.expasy.org/protscale/).

Mutagenesis of *GBA* gene and activity detection *in vitro*

Recombinant GCase was constructed by the pCDH system (System Biosciences, USA). We tested enzyme by detection of FLAG tag in the pCDH plasmid. The pCDH plasmid vector was digested with EcoRI and NotI for 2.5 h at 37°C and used as the target fragment. *GBA* and pCDH plasmid were ligated with NEB T4 DNA ligase (NEB#M0202) at 16°C overnight. Two loci carrying compound mutations of c.655A>G and c.1448T>C were successfully obtained using the KOD-Plus-Mutagenesis

Table 1: Primers for long fragment PCR in this study

Primers	Primer sequence	Size (bp)
GBA_EX1-5 (forward)	CCTAAAGTTGTCACCCATAC	2972
GBA_EX1-5 (reverse)	AGCAGACCTACCCTACAGTTT	
GBA_EX5-7 (forward)	GACCTCAAATGATATACCTG	2049
GBA_EX5-7 (reverse)	AGTTTGGGAGCCAGTCATTT	
GBA_EX8-11 (forward)	TGTGTGCAAGGTCCAGGATCAG	1682
GBA_EX8-11 (reverse)	ACCACCTAGAGGGGAAAGTG	

PCR: Polymerase chain reaction; GBA: Glucocerebrosidase.

Table 2: Primers for Sanger sequencing of *GBA* gene

Exons	Upstream	Downstream	Size (bp)	cDNA
GBA_EX1(2a)	cggaattacttgcagggcta	tctgtgccttgctcaaagag	288	1–193
GBA_EX2(3)	gtgggccttgtcctaatgaa	ctcaccccaaagttggtctc	363	194–281
GBA_EX3	ctcggcctcctaaagtgcta	tgaggacatccacagggaat	469	282–473
GBA_EX3-2(4)	caaggggtgaggaattttga	tatcagtacccagcgggaaa	394	282–473
GBA_EX4	taatcacacgggcacaggta	gctgaggcaggagaatcact	474	474–620
GBA_EX4-2(5)	taatcacacgggcacaggta	gctgaggcaggagaatcact	474	474–620
GBA_EX5(6)	aggagcccaagttcccttt	gttcagccattagcctccac	393	621–754
GBA_EX6	gacattttgtcccctgctgt	ctgatggagtgggcaagatt	473	755–927
GBA_EX6-2(7)	gacattttgtcccctgctgt	ctaggttgagggttgggaca	383	755–927
GBA_EX7	aggctgttctcgaactcctg	aggggaatggtgctctagg	498	928–1165
GBA_EX7-2(8)	aggctgttctcgaactcctg	gtttgggagccagtcatttg	448	928–1165
GBA_EX8(9)	aaaaatctccccaaacctctc	atcatggttccccagagttg	488	1166–1390
GBA_EX9(10)	tcacacccccaactccttag	tggggttttctgttgctacc	497	1391–1554
GBA_EX10(11)	agcaacagaaaaccccaatg	tgaatggagtagccaggtga	495	1555–1671
GBA_EX11(12)	gctctgctgttgtggtcgt	ctgggcttacgtcgctgt	356	1672–2324

GBA: Glucocerebrosidase.

Kit (TOYOBO, Osaka, Japan). Mutations were verified by sequencing. Recombinant target plasmid was transformed into competent *Escherichia coli* DH5α and amplified. Positive mono-bacterial colonies were picked and sequenced. Optimal corrected recombinant plasmids were kept. The two plasmids carrying the mutated loci and a recombinant plasmid without mutations were transfected into COS7 cells (African green monkey kidney fibroblast-like cell line, Cell Bank of Chinese Academy of Sciences) cultured in a 6 cm dish using polyethylenimine. Collected cells after 48 h and added cell lysis buffer to collected the protein. The 20 μl lysate was used to carry on Western analyses using FLAG tag antibody (DYKDDDDK tag) to verify recombinant plasmid could express in COS7 cells [Figure 1]. Then, other two groups' wild and mutated recombinant plasmids were expressed to test enzyme activity. After transfection, cells were collected in a new 2 ml centrifuge tube with 2 ml 1× phosphate buffered saline (PBS) buffer, and then washed with 1 ml 1× PBS buffer. The mixture was centrifuged (4000 *g* for 2 min) to separate cells from the supernatant. Deionized water was added to the cell pellets to make a homogenate by ultrasonication. The final concentration of the homogenate was 1–2 μg/μl determined by the Protein Quantitative Analysis Kit from Shanghai Biocolor BioScience & Technology Company (Shanghai, China). The activity of acid ß-GBA was measured using the artificial fluorogenic substrate 4-methylumbelliferyl-glucopyranoside (Sigma, USA). Mixtures of 0.2 mmol/L citrate-phosphate buffer (pH 5.5) and 0.6% (w/v) sodium taurocholate were added to the substrate for a final concentration of 5 mmol/L. Twenty microliters of the homogenates were mixed with 10 μl of substrate and incubated for 1 h at 37°C. The reaction was stopped by adding 200 μl glycine/$NaHCO_3$ buffer (pH 10.5). The fluorescence was read at an excitation wavelength of 365 nm and an emission wavelength of 450 nm using 4-MU to calibrate the fluorescence spectrometer.[10]

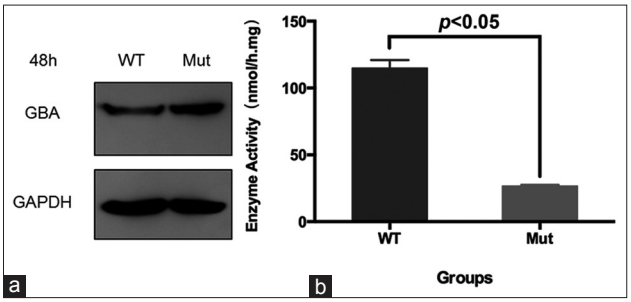

Figure 1: Contrastive activity analysis of GCase and mutation *in vitro*. (a) Early experiments verify rGCase expressed in COS7 cells. (b) Other two groups prove activity of the variant is about 77% lower than the wild. GBA: Glucocerebrosidase; WT: Wild group; Mut: Mutated group.

Results

DNA sequencing identifies a novel missense mutation in *GBA* gene

A novel heterozygous c.655A>G (p.T219A) mutation and a heterozygous c.1448T>C (p.L483P) were identified by Sanger sequencing in this patient. The c.655A>G (p.T219A) mutation was identified in his mother, while c.1448T>C (p.L483P) was identified in his father [Figure 2a]. The c.1448T>C (p.L483P) mutation has been previously reported.[10] We found that c.655A>G (p.T219A) mutation has never been reported neither in Chinese patient nor other ethnic populations.

GCase is conserved at two mutated loci

A comparison of the human wild-type GCase protein sequence (NP_001005742.1) with orthologs from rat, mouse, pig, sheep, and cow indicated that GCase was conserved at p.T219A and p.L483P [Figure 2b].

Structural prediction of mutant alleles

Protein structure models for the wild-type and c.655A>G mutant and c.1448T>C mutant proteins were generated by SWISS-MODEL and analyzed by PyMOL. The c.1448T>C (p.L483P) mutation[11,12] is located in domain 2 of GCase which can influence the catalytic activity of

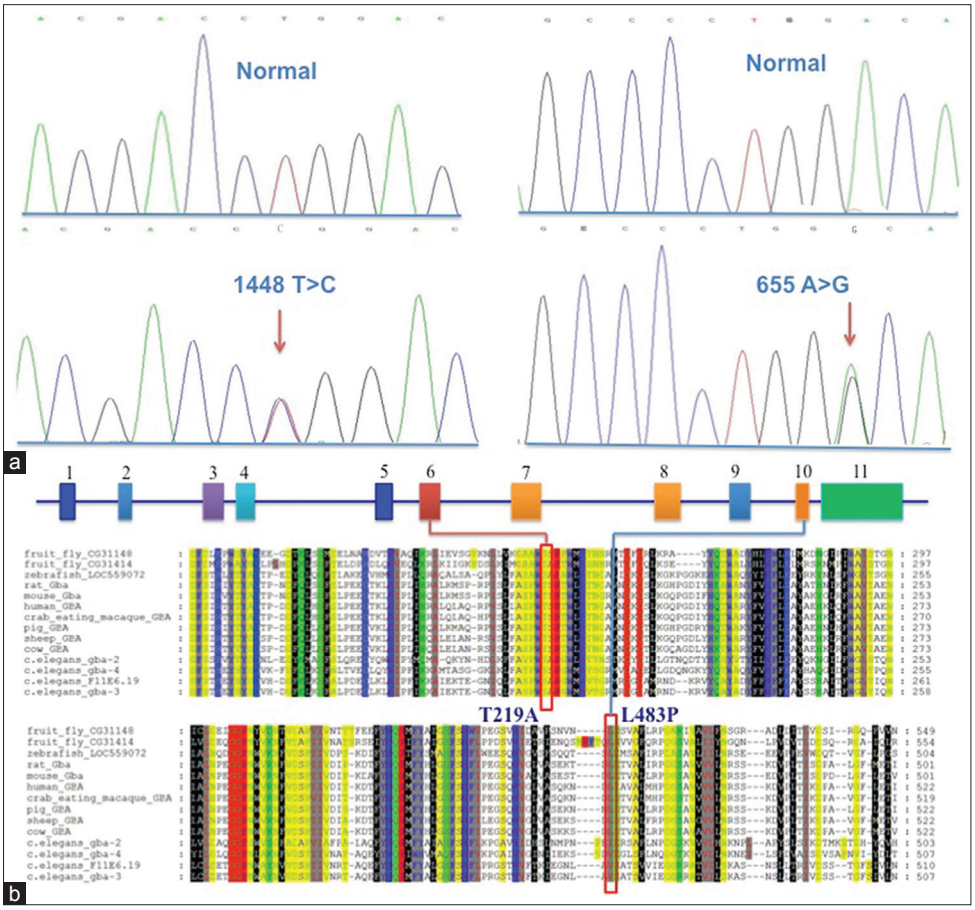

Figure 2: DNA sequencing identifies two novel variations in the *GBA* gene and evolutionary conservation prediction. (a) Normal and mutated sequence including p.T219A and p.L483P. (b) Amino acids on 219 and 483 are well conserved in human, rat, mouse, pig, sheep, and cow. GBA: Glucocerebrosidase.

GCase by changing the stability of the protein indirectly, and c.655A>G (p.T219A) is located in domain three which contains the catalytic center of the enzyme. p.T219A does not have an effect on enzyme activity directly because there are other acid, amino residues among p.T219, loop 1, 2, 3, and catalytic center E235, E340.[13,14] Residues filled between T219A and catalytic center are shown in Figure 3. T219 is near the surface of the protein and turns to be more hydrophobic when threonine changes to alanine. Hydroxyl of T219 could bind to K225 and C287 to form hydrogen bonding [Figure 4a and 4b]. Once mutation happened, the hydrogen bonding would disappear which lead to decrease of the stability of GCase in the area that results in activity declining [Figure 4c-4e].

GCase activity *in vitro*

After verifying that the recombinant plasmid could express GCase in COS7 cells [Figure 1a], we further investigated the effect of the mutation through other group tests. The activity of recombinant GCase analyzed by fluorescence spectrophotometry showed that the average activity of the wild-type was 114.69 nmol·h^{-1}·mg^{-1}, while the mutation was approximately 77% lower, at 26.37 nmol·h^{-1}·mg^{-1} [Figure 1b]. These data indicated that the heterozygous point mutation we identified in *GBA* gene led to a GCase mutation that reduced its activity. This led to the accumulation of glucocerebroside in the phagocyte system, which led to the formation of Gaucher cells, induced other manifestations of GD, and eventually led to GD.

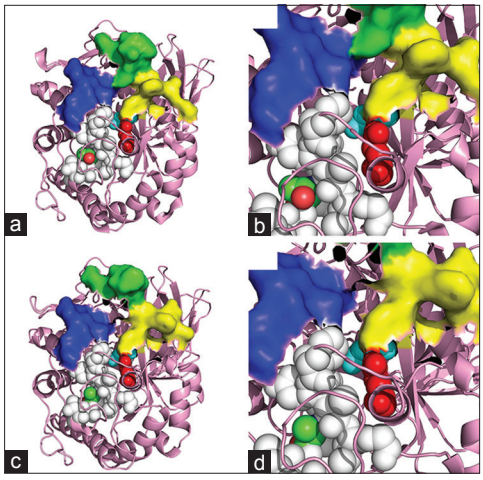

Figure 3: Residues are filled between T219A and catalytic center. (a and b) Integral and part view of the three-dimensional structure of GCase before mutation. (c and d) Integral and part view of the three-dimensional structure of GCase after mutation.

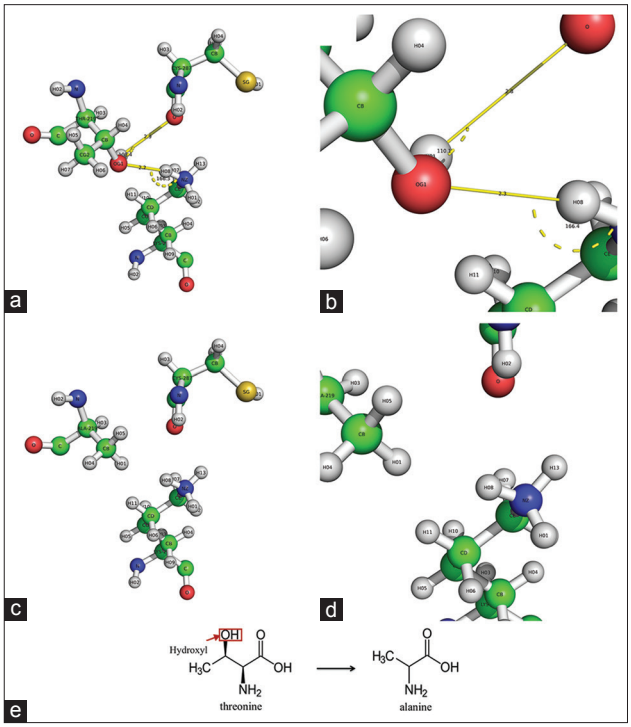

Figure 4: T219 provides hydroxyl to form hydrogen bonds with K225 and C287 but A219 cannot. (a and b) −OH (hydroxyl) of T219 can promote the formation of hydrogen bonds between T219 and C287 and K225. (c-e) −OH disappears after mutation (carbon atoms are green, nitrogen atoms are blue, oxygen atom are red, and hydrogen atoms are gray).

Discussion

It is estimated that the global morbidity of GD is between 1:40,000 or 50,000 and up to approximately 1:450 in the Ashkenazi Jew population.[15,16] The most effective treatment for these patients is enzyme replacement therapy. However, this treatment is expensive and not effective for patients with Types 2 and 3 GD.[17-20] Therefore, it is necessary to find a more effective therapy for GD.

A diagnosis of GD is suspected when there is an unexplained manifestation, such as hepatosplenomegaly, anemia, thrombocytopenia, or a bone marrow smear showing Gaucher cells.[21,22] The diagnosis can be confirmed by reduced GBA activity (usually <30% of normal) in peripheral blood leukocytes or skin fibroblasts. Mutations in *GBA* gene can be diagnosed by sequencing. The diagnosis of GD should be distinguished from leukemia, lymphadenoma, multiple myeloma, and other diseases.[21]

We diagnosed a pediatric patient from the Children's Hospital who was suspected of suffering from GD by identifying Gaucher cells in a bone marrow smear. This result was confirmed by a zymologic examination. Because the GBA activity was 1.9 nmol·h^{-1}·mg^{-1}, at least 70% lower than normal, the patient was diagnosed with GD.

At present, there have been more than 420 mutations identified in *GBA* gene according to the human gene mutation database. Genetic examinations of both the patient and his parents were necessary to ascertain the type of mutation, and Sanger sequencing identified heterozygous point mutations at c.655A>G (maternal disease haplotype) and c.1448T>C (paternal disease haplotype) of the patient. In combination with the clinical manifestations, we confirmed the patient's diagnosis of Type 1 GD. This compound heterozygous mutation was firstly found worldwide. Homozygous c.1448T>C (p.L483P) had been reported early in 1987 and was related to Type 2 GD.[23,24] Compared to patients with this compound heterozygous mutation, patients with homozygous c.1448T>C mutation developed earlier in 1–3 years old. Enzyme activity of GCase was 0 or 1.07 nmol·h^{-1}·mg^{-1} in Thai GD patients.[25]

The pathogenic predictions for the point mutations c.655A>G and c.1448T>C were made by Mutation Taster (http://www.mutationtaster.org), which indicated that it was a clear mutation type and that both were disease causing. Enzymatic assays revealed that the mutation reduced activity compared with the wild-type. Two groups of experiments were carried out in parallel. The tests showed that the mutation caused a statically significant loss of activity. We hypothesized that the genetic mutations caused a change in the protein sequence. We found changes in two amino acids that altered the primary structure of GCase, which will be discussed later. The prediction of evolutionary conservation was based on human, rat, mouse, pig, sheep, and cow. As expected, the two amino acids are well conserved in most mammals.

As is known to all, mature acid β-GBA is consists of two chains involving 497 amino acids according to RCSB database (http://www.rcsb.org/pdb/home/home.do). GCase has three domains including domain 1 (residues 1–29 and 384–414), domain 2 (residues 30–75 and 431–497), and domain 3 (residues 76–382 and 425–430) [Figure 3a].[11] The three-dimensional structures were predicted by SWISS-MODEL. Catalytic center E235 and E340 and could be seen in Figure 3c surrounded by loop 1 (residues 345–350), loop 2 (residues 393–399), and loop 3 (residues 312–319).[26] Mutant c.1448T>C is located in β-strand (D482–M489) and changed hydrophobic β-sheet of D443–D445. It was probably that T219A would influence catalytic center. The spatial relationships of residue 219, catalytic center, and three loops before and after mutation were analyzed by PyMOL [Figure 3]. It was obvious there were residues between T219 and catalytic center as well as A219. As a result, mutation T219A did not have an effect on E235 and E340, and the cap around them. The prediction of hydrophobic property of GCase by ProtScale was based on Kyte's method.[27] T219A became more hydrophobic and L483P more hydrophilic. This would directly lead to reaction changing of acid amino between mutation sites and others.

The novel mutation described here provided new evidence for the diagnosis of GD. Our laboratory was focused on the

mechanisms of neuronopathic GD and the development of therapeutic strategies. Epigenetic studies on mouse models of neuronopathic GD[28,29] are in progress, and the results will contribute to our understandings of GD.

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Conflicts of interest

There are no conflicts of interest.

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