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ORIGINAL ARTICLE

A common and two novel *GBA* mutations in Thai patients with gaucher disease

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Gaucher disease (GD) is an autosomal recessive disorder caused by mutations in the glucocerebrosidase (*GBA*) gene, leading to a deficiency of lysosomal β-glucosidase and accumulation of glycosphingolipids in macrophages. We studied five Thai families with GD (four with GD type 1 and one with GD type 2). Using long-template PCR, PCR using specific primers for the functional gene, direct sequencing of all coding regions of *GBA* and restriction enzyme digestions, all 10 mutant alleles were successfully identified. The common c.1448T>C (p.L483P or L444P) mutation was identified in 60% of mutant alleles. Of the two patients homozygous for the p.L483P (L444P) mutation, one died from hepatic failure at age 16 years and the other died from sepsis at age 12 years. This p.L483P (L444P) mutation was found in four different haplotypes, suggesting that it was a recurrent mutation, not caused by a founder effect. Two novel mutations, a missense (c.1204T>C, p.Y402H), and a termination codon mutation (c.1609T>C, p.X537A) were found. Studies to determine the molecular pathomechanism of the p.X537A mutation, the first of its kind in this gene, showed that it decreased the amount of protein being expressed and the enzymatic activity, while it was still correctly localized.
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Keywords: Gaucher disease; *GBA*; mutation spectrum; termination codon mutation

INTRODUCTION

Gaucher disease (GD; MIM 230800), the most prevalent lysosomal storage disorder, is caused by a deficiency of lysosomal enzyme glucocerebrosidase, or acid β-glucosidase, which normally hydrolyzes glucocerebroside to glucose and ceramide, leading to accumulation of glucocerebroside in macrophages. There are three clinical subtypes of GD, distinguished by the absence or presence and severity of neurologic complications. Type 1 (MIM 230800), or non-neuronopathic disease, does not involve the nervous system. Type 2 (MIM 230900), or acute-neuronopathic disease, is a fatal neurodegenerative disorder of infancy. Type 3 (MIM 231000), or chronic-neuronopathic disease, is a slowly progressive neurologic disease with survival into adulthood. Hepatosplenomegaly and bone lesions occur in all forms.[1]

The disease is an autosomal recessive disorder resulting from mutations in the glucocerebrosidase gene (*GBA*, MIM 606463) of ~7.5 kb, located on chromosome 1q21 and composed of 11 exons. A highly homologous ~5.5 kb pseudogene (*GBAP*, MIM 606463) that shares 96% exonic sequence homology is located 16 kb downstream from the functional gene.[2]

At least 291 different mutations have been identified in the *GBA* gene (the Human Genome Mutation Database, http://www.hgmd.cf.ac.uk/ac/index.php, February, 2013). The frequencies of specific mutated alleles vary in different populations. The most common mutation in the Ashkenazi Jewish, accounting for ~70% of mutations is N370S.[3] In this report, we studied the clinical, biochemical and molecular characteristics of five Thai families with GD.

MATERIALS AND METHODS

Subjects

Five unrelated Thai patients were diagnosed as having GD through clinical findings including hepatosplenomegaly and the presence of Gaucher cells in bone marrow aspiration. Based on the clinical findings, four unrelated patients were classified as type 1 and one as type 2. Consanguinity was denied in all families. With the exception of patient 1, who had an elder brother with similar clinical features and died at the age 18 months, others were sporadic cases. Glucocerebrosidase activity in leukocytes was determined by using a mixture of 4-methyl-umbelliferone-β-D-glucopyranoside as a substrate and

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fluorescence was measured with a Cecil spectrofluorometer using 450-nm emission and 365-nm excitation filters. The normal range of enzyme activity was determined in leukocytes of six normal Thais. Each patient's major clinical features and enzymatic activity are shown in Table 1.

Genotyping

After informed consent was obtained, high-molecular weight genomic DNA of all patients except patient 1 was extracted from peripheral blood leukocytes. Long-template PCR amplifying the entire *GBA* gene was performed using the Elongase Enzyme Mix (Invitrogen, Carlsbad, CA, USA) with primers GBA-F1 and GBA-R11 (Table 2). DNA was denatured for 30 s at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 68 °C for 7 min. The 6.6-kb product amplified by long-template PCR was gel purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA), then treated with ExoSAP-IT (USB Corporation, Cleveland, OH, USA), and sent for direct sequencing at Macrogen Inc., Korea. In addition, the genomic DNA of all patients including patient 1, whose high-molecular weight DNA was not available, was subjected to PCR amplification using primers designed to amplify each exon of the functional *GBA* gene but not the pseudogene (Table 2). PCR products were treated with ExoSAP-IT and sent for direct sequencing. Sequence analyses were performed by Sequencher 4.2. Samples with possible new variants were resequenced. The positions of mutations were compared with the *GBA* coding sequence (GenBank accession no. J03059.1).

The p.L483P (L444P), p.Y402H and p.X537A mutations were verified by restriction enzyme digestions of the patients' PCR products, using *Nci*I, *Rsa*I and *Cac*8I, respectively. Parents of patients with probable homozygous mutations for the p.L483P (L444P) mutation were also examined for the variants by restriction enzyme analysis. RNA from patient 5 who had the IVS6 mutation was extracted from peripheral blood leukocytes using QIAamp RNA blood mini kit (Qiagen). Reverse transcription was performed using ImProm-II reverse transcriptase (Promega, Madison, WI, USA) according to company recommendations. PCR amplification of the *GBA* cDNA covering the 3′-end of exon 4 through the termination codon to the 5- end of exon 11 was performed using primers cGBA4 and cGBA-R11 designed to amplify only the functional *GBA* cDNA, but not the cDNA of the pseudogene (Table 2).

Haplotype analysis of the p.L483P (L444P) allele

We used PCR-RFLP to detect the p.L483P (L444P) mutation in four additional patients with GD. Three were homozygous and one heterozygous for the mutation. DNA of these four cases, patients 1, 2 and 3, and available parents were PCR-amplified with primers as shown in Table 3 for previously described microsatellites (5GC3.2, ITG6.2, D1S2777, D1S1595 D1S2721). DNA of patient 4 was not available for this haplotype analysis. Genotyping was performed by Macrogen. Data were analyzed by GeneScan software (Applied Biosystem, Carlsbad, CA, USA).

Table 1 Clinical, biochemical and molecular data of the Thai GD patients

					Genotype[c]	
Patient no.	*Sex*	*Age at last follow-up or death*	*Type of GD; clinical features*[a]	*Enzyme activity*[b]	*DNA mutation*	*Protein change*
1	M	18 months	2; HS at age 7 months, regression of development, and death at age 18 months	NA	c.1448T>C/c.681T>C	p.L483P (L444P)/ p.N227K (N188K)[d]
2	F	16 years	1; HS at age 3 years, pancytopenia, splenectomy at age 7 years. Cirrhosis and hepatic failure, leading to death at age 16 years	1.07	c.1448T>C/c.1448T>C	p.L483P/p.L483P
3	F	12 years	1; HS at age 1 year, anemia and thrombocytopenia, splenectomy at age 4 years, chronic bone pain, and a pathologic fracture of right femoral neck at age 9 years. She died from sepsis at age 12 years	0	c.1448T>C/c.1448T>C	p.L483P/p.L483P
4	F	2 years	1; HS at age 7 months, pancytopenia, splenectomy at age 17 months	0.62	c.1448T>C/**c.1204T>C**	p.L483P/ **p.Y402H**
5	M	6 years	1; HS at age 3 years, anemia and thrombocytopenia.	1.29	c.IVS6-1 G>C/**c.1609T>C**	Not present/ **p.X537A**

Abbreviations: F, female; GD,Gaucher disease; HS, hepatosplenomegaly; M, male; NA, not available.
[a]All patients had HS and Gaucher cells in aspirated bone marrow. None received enzyme replacement therapy or bone marrow transplantation.
[b]Enzyme activity is expressed in nmol mg^{-1} protein per h, with the values of six Thai normal controls being 11.63 ± 4.97 (range: 5.97–19.58).
[c]Novel mutations are indicated in bold.

Table 2 PCR primers used to amplify genomic and complementary DNA of *GBA*

Gene/exon amplified	*Primer sequence (5′ to 3′)*	*Product size (bp)*
g. *GBA* and its pseudogene	GBA-F1: 5′-CCT AAA GTT GTC ACC CAT AC-3′ GBA-R11: 5′-ACC ACC TAG AGG GGA AAG TG-3′	6.6 and 4 kb
g. exons 1–2	GBA-F1: 5′-CCT AAA GTT GTC ACC CAT AC-3′ GBA-R2: 5′-CCA CCG AGC TGT AGC C-3′	1382
g. exons 3–4	GBA-F34: 5′-ATG TGT CCA TTC TCC ATG TCT TCA-3′ GBA-R34: 5′-ACG AAA AGT TTC CGT GGC TCT-3′	523
g. exons 5–6	GBA-F56: 5′-GAC CTC AAA TGA TAT ACC TG-3′ GBA-R56: 5′-GAA AGG TCA TGA ATG A-3′	1690
g. exon 7	GBA-F7: 5′-TCA AGA CCA ATG GAG CGG TG-3′ GBA-R7: 5′-AGT TTG GGA GCC AGT CAT TT-3′	953
g. exon 8	GBA-F8: 5′-GTT GCA TTC TTC CCG TCA CC-3′ GBA-R8: 5′-CTG GAC AGG AAG GGC TTC TG-3′	367
g. exons 9–11	GBA-F911: 5′-AAC CAT GAT TCC CTA TCT TC-3′ GBA-R911: 5′-ACC ACC TAG AGG GGA AAG TG-3′	1249
c. 3-end of exon 4 to 5′-end of exon 11	cGBA4: 5′-TGA CAG ATG CTG CTG CTC TC-3′ GBA-R11: 5′-ACC ACC TAG AGG GGA AAG TG-3′	1409
c. entire coding region of the X537A mutant	GBA-XhoI-F: 5′-ATC TCG AGC ATC TAA TGA CC-3′GBA-EcoRI-R: 5′-ATG AAT TCT GAG TCAC CCA A-3′	1797

Abbreviations: c, complementary DNA; g, genomic DNA; GBA, glucocerebrosidase.

[d]L444P and N188K are the names that follow the common nomenclature and do not include the 39-residue signal peptide.

Functional analysis of the p.X537A mutation

For glucocerebrosidase activity analysis, RNA of normal controls and patient 5 was extracted from peripheral blood leukocytes with QIAamp RNA blood mini kit (Qiagen), then reverse-transcribed and amplified by PCR using primers GBA-XhoI-F and GBA-EcoRI-R (Table 2) to add the *Xho*I and *Eco*RI sites at the 5′ and 3′ ends, respectively. The amplified cDNA was cloned into the pGEM-T Easy Vector System (Promega) and subsequently into the pcDNA3.1 expression vector (Invitrogen), and sequenced. These constructs were transiently transfected into COS-7 (fibroblast) cells, using Lipofectamine 2000 (Invitrogen). After 24-hr transfection, cells were centrifuged and sonicated in dH_2O. Protein concentration was determined by BCA protein assay (Micro BCA Protein Assay Kit, PIERCE, Rockford, IL, USA) and the glucocerebrosidase activity was measured by fluorimetric assay using 4-methyl-umbelliferone-β-D-glucopyranoside.

For protein expression analysis, the p.N409S (N370S) mutation, a positive control for the enzyme mislocalization to the ER,[4] was generated using QuikChange Site-Directed Mutagenesis Kit (Stratagene). The *GBA* clones were confirmed by direct sequencing. Cells transfected with the wild-type, p.X537A and p.N409S (N370S) *GBA* were immunoblotted to measure the level of glucocerebrosidase. Protein extracts were prepared from cell pellets harvested in lysis buffer with freshly added protease inhibitors, subjected to 10% polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blotted with antibodies to glucocerebrosidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and actin (Santa Cruz Biotechnology), a loading control.

Table 3 Markers and primers to determine haplotypes

Marker	*Forward primer (5′ to 3′)*	*Reverse primer (5′ to 3′)*
D1S2777	5′-GCACCACGGAACTCCAGTAT-3′	5′-CACCACTGTGCCCAGCTAAT-3′
D1S2721	5′-TTGCTCGGCCAGAGTCT-3′	5′-ACGCATCACACCTGGCTAGT-3′
D1S1595	5′-ATGGTATGAACCTGGAGGTG-3′	5′-GGCAGATAAAAGGACTGCAA-3′
ITG6.2	5′-CACATGAGGTCAGGTGTTTG-3′	5′-GCAAAGGAGTGGTGAACTTC-3′
5GC3.2	5′-TTCAATCGCCCCCATCCACC-3′	5′-TCAGAGCCCTTCCTCAAG-3′

For subcellular localization analysis, the COS-7 cells transfected with the wild-type and p.X537A *GBA* were analyzed by immunofluorescence staining. Cells were grown directly on Lab-Tek II chamber slides, and fixed 24–48 h after transfection with cold methanol. Cells were incubated with rabbit polyclonal immunoglobulin G antibodies to glucocerebrosidase (Santa Cruz Biotechnology), and mouse monoclonal immunoglobulin G1 antibodies to LAMP2 as a lysosomal marker (Abcam, Cambridge, UK). These cells were subsequently analyzed with Carl Zeiss LSM 700 confocal microscope.

RESULTS

The clinical, biochemical and mutational profiles of patients are summarized in Table 1. Four patients were classified as GD type 1 and one as type 2. Among patients with type 1 GD, the onset of hepatosplenomegaly ranged from 7 months to 7 years old. All patients whose leukocytic glucocerebrosidase activities were determined had no or markedly low activities ($<1.07\,nmol\,mg^{-1}$ protein per hour) compared with $11.63 \pm 4.97\,nmol\,mg^{-1}$ protein per hour in Thai controls.

We successfully identified all 10 mutant alleles with either long-template PCR or PCR using specific primers for the functional *GBA* gene (Table 2). Both methods gave the same results. Four patients had the p.L483P (L444P) mutation. Patients 1 and 4 were compound heterozygotes (Figure 1a lower panel, lanes 5 and 6, respectively). Patients 2 and 3 were homozygotes (Figure 1a lower panel, lanes 4 and 3, respectively). The parents of patients 2 and 3 denied consanguinity, and were all heterozygous for the c.1448T>C (p.L483P) mutation. Patient 2 died from hepatic failure at age 16 years. Patient 3 had thrombocytopenia requiring splenectomy at age 4, fracture of her right femoral neck at age 9 and died from sepsis at age 12. This p.L483P (L444P) mutation was found in four different haplotypes (Table 4).

Two novel mutations were identified. A novel non-synonymous variant, c.1204T>C (p.Y402H), in exon 8 was found in patient 4 (Figure 1b middle panel and lower panel) and a novel termination

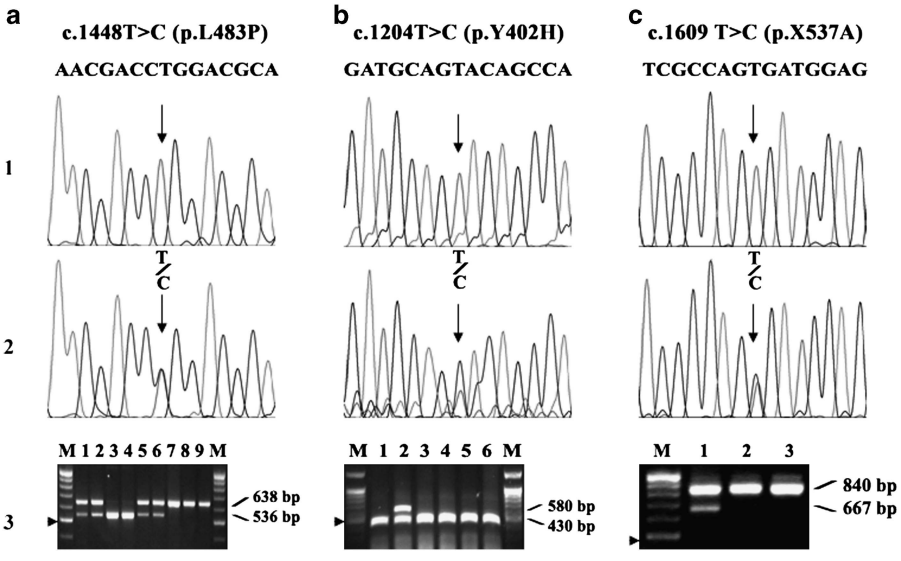

Figure 1 Mutation analysis of the common p.L483P (L444P) mutation (column **a**) and the novel mutations, p.Y402H (column **b**) and p.X537A (column **c**). Upper and middle panels, electropherograms of the wild-type and heterozygous mutant alleles, respectively. Lower panel, restriction enzyme digestion analysis. M is a 100-bp marker. The arrow heads indicate the 500-bp bands. In column **a**, the p.L483P (L444P) mutation creates an *Nci*I restriction site. Therefore, the 638-bp PCR products of the mutant allele of individuals homozygous (lanes 3 and 4) and heterozygous (lanes 1, 2, 5, and 6) for the mutation were digested into 536-bp and 102-bp (not shown) products. In column **b**, the p.Y402H mutation in patient 4 (lane 2) eliminates the *Rsa*I site, leaving the 580-bp product intact. The 580-bp product was digested into 430 and 150-bp (not shown) products of the other allele. In column **c**, the p.X537A mutation in patient 5 (lane 1) creates a *Cac*8I site. Therefore, the 840-bp product of the mutant allele was digested into 667 and 173-bp (not shown) products. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Table 4 Forms and frequencies of haplotypes of the p. L483P (L444P) mutation in Thai patients

Patient	*Mutation*	*ITG6.2*	*5GC3.2*	*D1S2777*	*D1S1595*	*D1S2721*
1	p.L483P[b]	314	219	264	279	232
	p.N227K	314	221	264	279	236
2	p. L483P[a]	318	217	268	279	238
	p.L483P[a]	318	217	268	279	238
3	p.L483P[a]	318	217	266/270	279/283	234/236
	p.L483P[b]	314	219			
4	p.L483P	ND	ND	ND	ND	ND
	p.Y402H	ND	ND	ND	ND	ND
6	p.L483P[c]	306	217	268	279	240
	p.L483P[c]	306	217	268	279	240
7	p.L483P[a]	318	217	268	283	238
	p.L483P[a]	318	217	268	283	238
8	p.L483P[d]	322	217	268	279	238
	p.L483P[b]	314	219	264	279	232
9	p.L483P[a]	318	217	268	279	238
	?	314	221	264	279	236

	n	*Haplotype frequency*
Haplotype a	6	0.50
Haplotype b	3	0.25
Haplotype c	2	0.17
Haplotype d	1	0.08

Abbreviation: ND, not determined.
L483P (L444P) mutation in Thai patients.

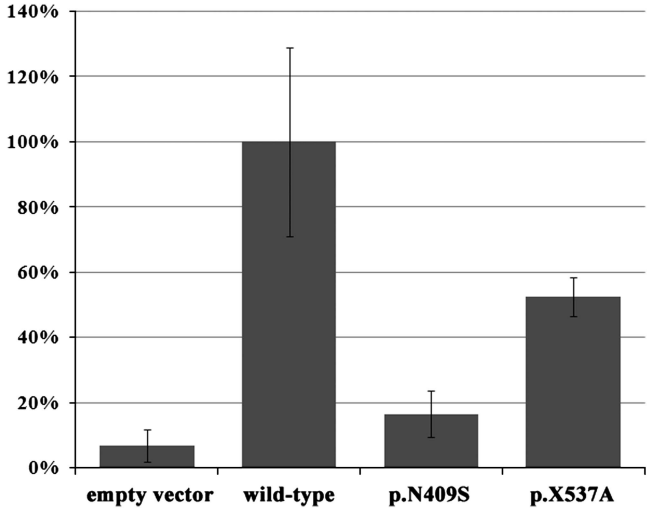

Figure 2 Glucocerebrosidase (GBA) activity analysis of the p.X537A mutant. Enzyme activity was measured on extracts of cells transfected with the empty pcDNA3.1 vector (empty vector), wild-type *GBA* (wild-type), p.N409S mutant (p.N409S) and p.X537A mutant (p.X537A). Enzyme activity from cells transfected with the wild-type *GBA* was used as a baseline.

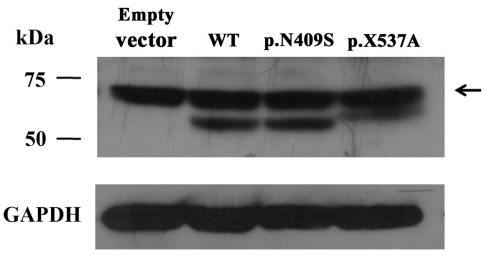

Figure 3 Glucocerebrosidase (GBA) expression analysis of p.X537A by western blotting. Top panel: Empty vector, wild-type (WT), p.N409S, p.X537A = cells transfected with the empty pcDNA3.1 vector, wild-type *GBA*, p.N409S mutant and p.X537A mutant, respectively. Arrow = a non-specific band. Lower panel: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) loading control.

codon mutation, c.1609T>C (p.X537A) (Figure 1c middle panel and lower panel), was found in patient 5. The boy was heterozygous for a splice site mutation, IVS6-1G>C. Sequencing of his cDNA revealed only the sequence of the p.X537A allele with no detectable level of the IVS6-1G>C transcript (Figure 1c lower panel). Expression of the p.X537A mutant and the wild-type clones in COS-7 cells demonstrated that the mutation reduced the residual glucocerebrosidase enzyme activity with respect to controls (Figure 2) and reduced the protein expression level (Figure 3), but did not significantly change the protein subcellular localization in mammalian cells (Figure 4).

DISCUSSION

The presence of a highly homologous pseudogene sequence and several types of mutations including large recombinant alleles or deletions has made mutation analysis of *GBA* challenging.[5] Using solely conventional PCR and SSCP or PCR-based mutation-detection techniques to screen for specific mutations might result in a significant number of unidentified mutant alleles.[6] Therefore, in this study, direct mutation detection was performed by sequencing the entire coding region in all patients either by long-template PCR or PCR using specific primers for the functional gene. With these methods, all ten mutant alleles were identified in our five patients (see Table 1).

Patient 1 was compound heterozygous for p.L483P/p.N227K (L444P/N188K). The N188K mutation is a rare mutation previously found in patients with type 2 GD.[7] Sequencing electropherogram of patients 2 and 3 showed only the c.1448T>C (p.L483P) mutation, which could be homozygous or hemizygous. Therefore, the genotypes of their parents were studied and revealed that all were heterozygous for the p.L483P (L444P) mutation, suggesting that patients 2 and 3 were homozygous for the mutation. p.L483P (L444P) is the most common mutant allele in the Thai patients with GD studied, accounting for 60% of mutant alleles. Notably, because conventional PCR method could not distinguish the isolate p.L483P (L444P) mutation and the p.L483P (L444P) as a part of a recombinant allele carrying additional pseudogene sequence alterations, further evaluation by other techniques such as direct sequencing to establish the presence or absence of a complex allele should be considered.[8,9] The p.L483P (L444P) mutation occurs with significant frequencies in various populations, including 41% of mutant alleles in the Japanese,[6] and 2.84% in the Jewish,[10] At the age of death at 16 years in patient 2 and 12 years in patient 3, both patients with homozygous p.L483P (L444P) did not show primary neurological manifestations, and, therefore, were categorized as type 1. A previously reported Thai patient with homozygous for the p.L483P (L444P) mutation was described to have a 'Norbottnian-like' phenotype, with mild neurological involvement.[11] However, 15 of 19 Caucasian patients homozygous for the p.L483P (L444P) had neuronopathic GD.[12] In addition, 9 of 12 Japanese patients with this genotype who were originally diagnosed with non-neuronopathic GD, developed neurological signs/symptoms during follow-up.[13] Patient 2 died from cirrhosis and hepatic failure.

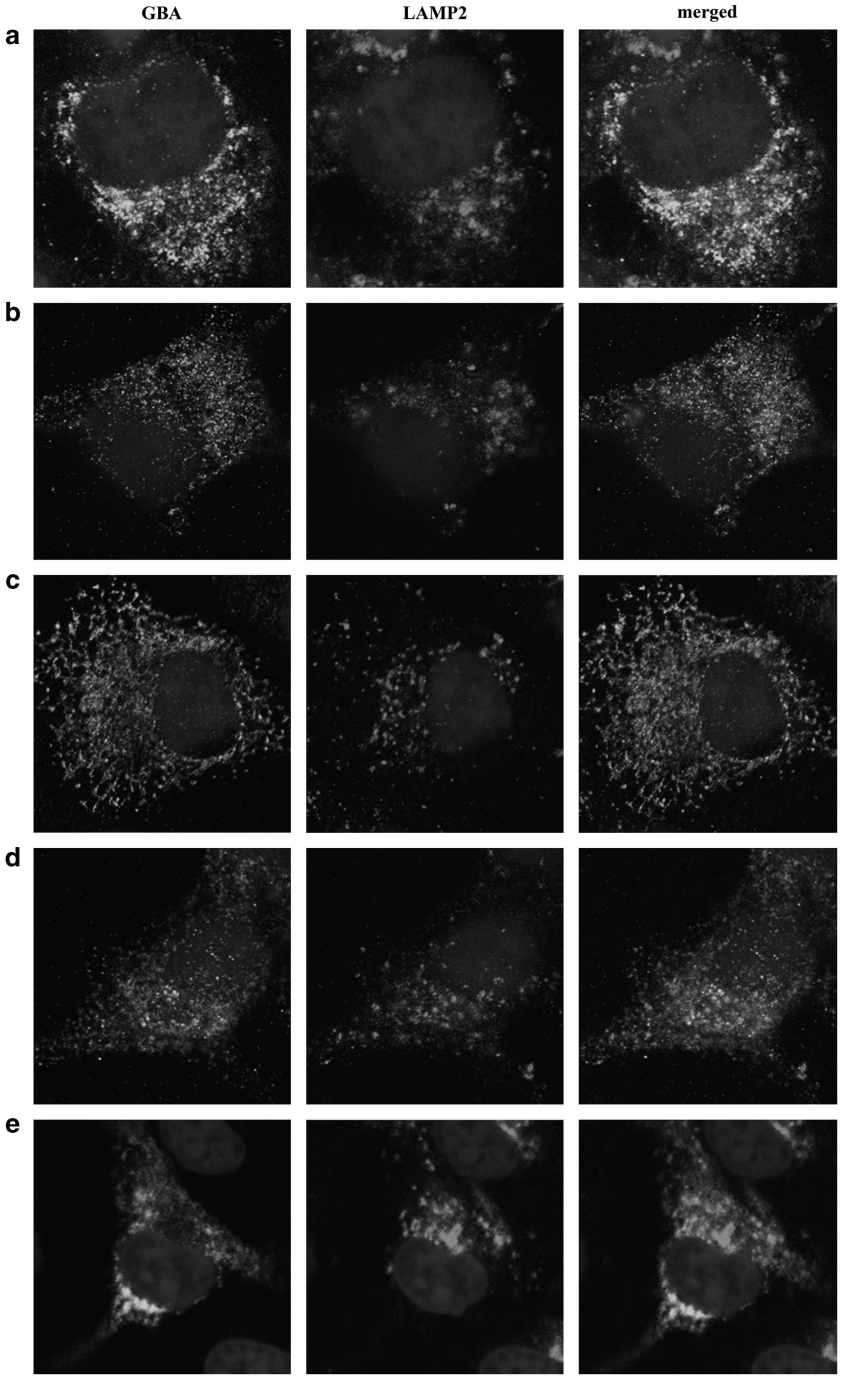

Figure 4 Subcellular localization analysis of p.X537A mutant by immunofluorescence confocal microscopy. (**a**, **b**) = wild-type glucocerebrosidase (GBA), (**c**, **d**) = p.X537A mutant, E = p.N409S. Green = GBA, red = LAMP2 (a lysosomal marker), blue = nuclei counterstained with 4′,6-diamidino-2-phenylindole. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Owing to no other identifiable causes of cirrhosis, this could be a complication of GD. There are several previous reports of GD patients dying from cirrhosis.[14,15] This p.L483P (L444P) mutation was found in four different haplotypes, suggesting that it was a recurrent mutation, not caused by a founder effect.

Two novel mutations including a missense (c.1204T>C, p.Y402H) and a mutation in the termination codon (c.1609T>C, p.X537A) were identified. A splice site mutation previously reported in a Thai patient with type 3 GD,[11] the IVS6-1G>C change, was compound heterozygous with the c.1609T>C (p.X537A) in patient 5. Sequencing of his cDNA showed no skipping around IVS6 and only the C at position 1609 (Figure 1C lower panel), suggesting that it contained only the sequence of the p.X537A allele. It is probable that the IVS6-1G>C is pathogenic due to the absence of its mRNA, which is most likely from a splicing defect, leading to a premature stop codon and degradation of its mRNA via a nonsense mediated mRNA decay.[16] The c.1609T>C (p.X537A) mutation is the first identified termination codon mutation in the *GBA* gene and expected to result in the addition of 15 more amino acids before coming to a new stop. Functional studies have suggested that the mutation decreases the

amount of protein being expressed and the enzymatic activity, while it is still correctly localized. Termination codon mutations have been observed in many disorders, including Wiskott–Aldrich syndrome.[17]

In conclusion, we report five Thai patients with GD. A common mutation, p.L483P (L444P), accounting for 60% of mutant alleles, and two novel mutations, c.1204T>C (p.Y402H) and c.1609T>C (p.X537A), were identified. Phenotypes of the two patients homozygous for p.L483P (L444P) were severe; both died in their teens. Long-term follow-up is necessary in determining the disease severity associated with the novel mutations.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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