

Identification of Seven Novel Mutations in the Acid Alpha-glucosidase Gene in Five Chinese Patients with Late-onset Pompe Disease

Hua-Xu Liu¹, Chuan-Qiang Pu¹, Qiang Shi¹, Yu-Tong Zhang¹, Rui Ban^{1,2}

¹Department of Neurology, Chinese People's Liberation Army General Hospital, Beijing 100853, China

²School of Medicine, Nankai University, Tianjin 300071, China

Abstract

Background: Pompe disease is a rare lysosomal glycogen storage disorder linked to the acid alpha-glucosidase gene (GAA). A wide clinical and genetic variability exists between patients from different ethnic populations, and the genotype-phenotype correlations are still not well understood. The aim of this study was to report the clinicopathological and genetic characteristics of five Chinese patients with late-onset Pompe disease (LOPD) who carried novel *GAA* gene mutations.

Methods: Clinical and pathological data of patients diagnosed with glycogen storage disease at our institution from April 1986 to August 2017 were collected, and next-generation sequencing of frozen muscle specimens was conducted.

Results: Of the five patients included in the study, the median disease onset age was 13 years, with a median 5 years delay in diagnosis. The patients mainly manifested as progressive weakness in the proximal and axial muscles, while one patient developed respiratory insufficiency that required artificial ventilation. In muscle biopsies, vacuoles with variable sizes and shapes appeared inside muscle fibers, and they stained positive for both periodic acid-Schiff and acid phosphatase staining. Ten *GAA* gene mutations, including seven novel ones (c.796C>A, c.1057C>T, c.1201C>A, c.1780C>T, c.1799G>C, c.2051C>A, c.2235dupG), were identified by genetic tests.

Conclusions: The seven novel *GAA* gene mutations revealed in this study broaden the genetic spectrum of LOPD and highlight the genetic heterogeneity in Chinese LOPD patients.

Key words: Alpha-glucosidase; DNA Mutational Analysis; Genetic Heterogeneity; Glycogen Storage Disease Type II

INTRODUCTION

Pompe disease (glycogen storage disease Type II, acid maltase deficiency, and OMIM #232300) is a rare, progressive, autosomal recessive metabolic disorder caused by a deficiency of the lysosomal enzyme acid alpha-glucosidase (GAA). The estimated disease incidence of Pompe disease at birth is 1 in 40,000,^[1] but other studies showed that the incidence might be underestimated with 1: 8684 in Austria^[2] and 1: 11,987 in Taiwan, China.^[3]

According to symptom onset age and disease severity, Pompe disease is classified into two forms: the infantile-onset form and the late-onset form (childhood-, juvenile-, and adult-onset forms). Patients with infantile-onset Pompe disease have severe hypotonia and muscle weakness with cardiac involvement in the first few months of life that induce cardiorespiratory failure and death before 1 year of age. In

contrast, the phenotype of late-onset Pompe disease (LOPD) is less severe and characterized by slowly progressive myopathy with or without respiratory impairment.

The *GAA* gene is mapped to the human chromosome 17q25.2–q25.3. It contains 20 exons with the first amino acid encoded in exon 2 and the last in exon 20. Pathogenic sequence variations are distributed across all 19 coding genes, but the catalytic barrel gene (c.1039-c.2454) contains

Address for correspondence: Dr. Chuan-Qiang Pu, Department of Neurology, Chinese People's Liberation Army General Hospital, Beijing 100853, China
E-Mail: pucq30128@sina.cn

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the highest percentage of pathologically severe mutations.^[4] To date, over 550 different mutations have been described worldwide (<http://www.pompecenter.nl>, updated May 2016). There is a marked genotype heterogeneity among LOPD patients from different ethnics and areas, but to the best of our knowledge, only 18 articles about *GAA* gene mutations in Chinese LOPD patients have been published with 32 novel mutations in total.^[3,5-21] To further expand the genetic spectrum and understand the genotype-phenotype correlations, herein we report five Chinese LOPD patients with seven novel *GAA* gene mutations.

METHODS

Ethical approval

As a retrospective study for the clinicopathological features of LOPD patients, muscle specimens used for genetic analysis were obtained from previous clinical work and data analysis was performed anonymously. This study was approved by the Ethics Committee of Chinese People's Liberation Army General Hospital. Patient consent was exempt by the Ethics Committee due to the retrospective nature of this study.

Patients

Five patients diagnosed with LOPD at our institution from April 1986 to August 2017 were included in this study. They were from unrelated families, and none had consanguineous parents. The confirmatory diagnosis was based on clinical characteristics, muscle pathology, and genetic tests. Detailed general history, clinical features, and laboratory data were collected.

Muscle biopsy and pathology

All five patients had open muscle biopsies, three in musculus biceps brachii and two in quadriceps femoris. After removal, muscle samples were fixed in glutaraldehyde, frozen in liquid nitrogen, and then, stored in -80°C . Serial muscle sections ($5\ \mu\text{m}$ thick) were stained for hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), oil red O (ORO), modified Gomori trichrome (MGT), nicotinamide adenine dinucleotide dehydrogenase (NADH), nonspecific esterase (NSE), acid phosphatase (ACP), and adenosine triphosphatase (ATPase) after preincubation in pH 4.3, 4.5, and 10.6. Morphometric evaluations of stained muscle sections were performed with light microscopy (Olympus Corporation, Japan).

Genetic analysis

A gene panel that contains 142 neuromuscular disorder-related genes, including 15 genes responsible for glycogen storage disease, was used for next-generation sequencing. Genomic DNA was extracted from frozen muscle specimens using QIAmp DNA Blood Kits (Qiagen, Hilden, Germany). The DNA probes were designed to bind exons and 50 bp flanking intronic sequences. The final enriched DNA libraries were sequenced on Illumina HiSeq 2500 platform (Illumina, the United States of America) according to the manufacturer's protocol. Variants were further confirmed by Sanger sequencing.

The novel mutations were searched in the Single Nucleotide Polymorphism database (dbSNP, <https://www.ncbi.nlm.nih.gov/projects/SNP/>, National Institutes of Health, USA), the 1000 Genomes database (<http://www.internationalgenome.org>, the International Genome Sample Resource), Exome Sequencing Project (ESP6500, <http://evs.gs.washington.edu/EVS/>, National Heart, Lung and Blood Institute, USA), Exome Aggregation Consortium (ExAC, <http://exac.broadinstitute.org/>, Broad Institute, USA), the Genome Aggregation Database (gnomAD, <http://gnomad.broadinstitute.org/>, international coalition of investigators), and the clinVar database (<https://www.ncbi.nlm.nih.gov/clinvar/>, National Institutes of Health, USA) and the in-house database for normal healthy controls. Pathogenicity of novel missense mutations was predicted using bioinformatics software programs such as SIFT (<http://sift.bii.a-star.edu.sg/>, Genome Institute of Singapore, Singapore), Mutation Taster (<http://www.mutationtaster.org/>, Charité Universitaetsmedizin Berlin, Germany),^[22] and PolyPhen2 (<http://genetics.bwh.harvard.edu/pph2/>).^[23]

Statistical analysis

Descriptive statistics were used to analyze the clinical and laboratory data. Proportions were calculated for categorical variables (i.e. gender). Summary statistics (median, range) were calculated for continuous measures (i.e., age and disease course). The median was chosen as the descriptive measure for the disease onset age, diagnostic delay, and the age at diagnosis. SPSS 17.0 software (the International Business Machines Corporation, USA) was used for statistical analysis.

RESULTS

Clinical manifestations

The study involved five patients, three were males and two were females. The disease onset age ranged from 1 to 19 years with a median of 13 years. The median age at diagnosis was 22 years (range 16–42 years) with a median disease course of 5 years (range 3–41 years). None of the patients had a positive family history. Progressive skeletal muscle weakness was the initial and cardinal symptom, which mainly affected the proximal (100%) and axial (80%) muscles with predominant involvement of the lower limbs. One patient suffered from progressive respiratory insufficiency within a month and required artificial ventilation. Two patients had scoliosis, and one patient had both lordosis and kyphosis. Three patients had weakness in the anterior cervical muscles. The disease onset age for the four patients who had axial muscle weakness ranged from 1 to 13 years while the patient without axial muscle involvement had a later onset of initial symptoms at 19 years. Detailed clinical features were summarized in Table 1.

Laboratory data

Creatine kinase (CK) and lactic dehydrogenase (LDH) levels were tested in all patients at the time of diagnosis. Four patients (80%) had mild-to-moderate elevations

in CK levels, while three patients (60%) had mild elevated LDH levels. The median levels for CK and LDH were 1168.3 U/L (range 73.5–1652.0 U/L; normal <200.0 U/L) and 331.8 U/L (range 151.0–610.0 U/L; normal 40.0–250.0 U/L), respectively. All patients had myogenic damage in electromyography examinations.

Muscle biopsies

Muscle biopsies were performed in all five patients. H and E staining showed vacuoles with variable sizes and shapes inside muscle fibers which stained positive in PAS and exhibited strong reactions for lysosomal ACP staining. Blue basophilic particles were found inside the vacuoles in all five cases (100%). Internal nuclei and endomysial fibrosis were observed in three patients (60%), while fiber hypertrophy and split appeared in two patients (40%). No RRF (ragged red fiber) was found in GMT staining. ORO staining showed no lipid storage for all five patients. Hyperchromatic necrotic fibers could be seen in NADH and NSE staining. In ATPase staining, two patients (40%) had an even distribution of Type I and Type II fibers, while Type II fibers were dominant in two patients (40%) and Type I fibers were dominant in one patient (20%). Vacuolization solely affected Type II fibers in two patients (40%), and affected both fiber types in the other three patients (60%).

Genetic analyses

All five patients underwent genetic tests and were found to have compound heterozygous mutations in *GAA* gene. Ten *GAA* gene sequence variants were detected, including three reported mutations (c.1309C>T, c.2237G>A, and c.2238G>C) and seven novel mutations. Nine of the ten mutations (90%) were found in the catalytic barrel (c.1039-c.2454) except mutation c.796C>A. Each mutation was detected in only one patient with an allele frequency of 10% (1/10). The seven novel mutations

were composed of one nonsense mutation (c.1057C>T), one frameshift mutation (c.2235dupG), and five missense mutations (c.796C>A, c.1201C>A, c.1780C>T, c.1799G>C, and c.2051C>A). Detailed molecular information was summarized in Table 2.

None of the seven novel mutations were found in the SNP138, 1000 Genomes, ESP6500, ExAC, gnomAD, ClinVar databases or the in-house database for normal healthy controls. By conservation analysis, the mutation p.P266T (c.796C>A) was proved to be less conserved among different species, while the other four novel missense mutations were highly conserved [Figure 1]. In silico analysis, the c.796C>A mutation was predicted to be benign, and the other four missense mutations were predicted to be deleterious. The nonsense mutation c.1057C>T (p.Q353X) was presumed to be deleterious because it caused a premature termination codon upstream of the catalytic site and could lead to the formation of a truncated protein and loss of GAA enzyme function. The frameshift mutation c.2235dupG (p.L745fs) could also introduce a premature stop codon and was thus predicted to be deleterious.

DISCUSSION

Here, we provide clinicopathological and genetic analysis in a cohort of five LOPD patients with novel *GAA* gene mutations diagnosed at our institution over the past 30 years. The dominant clinical manifestation for the five patients was muscle weakness in the proximal extremities (100%) and axial muscles (80%), which is consistent with some previous reports.^[24,25] However, in 2012, a study of 94 patients with Pompe disease represented a different pattern of muscle involvement. In that study, the strength of the quadriceps muscle was reduced in only 55% of patients, but the shoulder abductors, abdominal muscles, paraspinal muscles, and

Table 1: Clinical and pathological features of five LOPD patients

Number	Gender	Age of onset (years)	Age of diagnosis (years)	Initial symptoms	CK (U/L)	EMG	Muscle biopsy
1	Male	1	42	Muscle weakness	1325.6	Myogenic damage	Vacuolar myopathy
2	Male	1	18	Muscle weakness of lower extremities	1168.3	Myogenic damage	Vacuolar myopathy
3	Female	19	22	Muscle weakness	73.5	Myogenic damage	Vacuolar myopathy
4	Female	13	16	Muscle weakness of lower extremities	1652.0	Myogenic damage	Vacuolar myopathy
5	Male	13	18	Muscle weakness	586.2	Myogenic damage	Vacuolar myopathy

LOPD: Late-onset Pompe disease; CK: Creatine kinase; EMG: Electromyography.

Table 2: The *GAA* gene mutations of five LOPD patients

Number	Allele 1	Location 1	Transcript 1	Effect 1	Allele 2	Location 2	Transcript 2	Effect 2
1	c.1201C>A	Exon 8	p.Q401K	Unknown	c.1057C>T	Exon 6	p.Q353X	Unknown
2	c.2238G>C	Exon 16	p.W746C	Potentially mild	c.2235dupG	Exon 16	p.L745fs	Unknown
3	c.1309C>T	Exon 8	p.R437C	Less severe	c.2051C>A	Exon 15	p.P684Q	Unknown
4	c.2237G>A	Exon 16	p.W746X	Very severe	c.796C>A	Exon 4	p.P266T	Unknown
5	c.1799G>C	Exon 13	p.R600P	Unknown	c.1780C>T	Exon 13	p.R594C	Unknown

GAA: Acid alpha-glucosidase; LOPD: Late-onset Pompe disease.

	p.P266T	p.Q401K	p.R594C	p.R600P	p.P684Q
<i>Homo sapiens</i>	AEHLSPLMLS	QWNDLDYMDS	RGTRPFVISR	RGTRPFVISR	PQEPYSFSEP
<i>Pan troglodytes</i>	AEHLSPLMLS	QWNDLDYMDS	RGTRPFVISR	RGTRPFVISR	PQEPYSFSEP
<i>Equus caballus</i>	AEHLGFLMLN	QWNDLDYMDA	RGVRRPFVISR	RGVRRPFVISR	PQEPYRFSET
<i>Chinchilla lanigera</i>	AEHLSPLMLR	QWNDLDYMDA	RGTRPFVISR	RGTRPFVISR	PQEPYRFSEP
<i>Bison</i>	AEHLGSLMLS	QWNDLDYMDA	RGMRRPFVISR	RGMRRPFVISR	PQEPYRFSET
<i>Canis lupus familiaris</i>	AEHLGSLMLS	QWNDLDYMDA	RGTRPFVISR	RGTRPFVISR	PQEPYRFSAT
<i>Gallus gallus</i>	GERLTPLILD	QWNDLDYMDA	RGTRPFVISR	RGTRPFVISR	PQEPYAFSLA
<i>Danio rerio</i>	GEHYTPTILD	QWNDLDYADQ	KKTRPFVLSR	KKTRPFVLSR	PQEPYVFSQR
<i>Schizosaccharomyces pombe</i>	AETIHGLRLG	FWSIDIDYMEK	PNERPFILSR	PNERPFILSR	SQEPYTWS
<i>Capra hircus</i>	AEHLGSLMLS	QWNDLDYMDA	RGTRPFVISR	RGTRPFVISR	PQEPYRFSET

Figure 1: Conservation analyses of the five novel missense mutations in different species.

gluteus muscles were affected in more than 80% of all patients.^[26] The differences could come from the different methods to measure muscle strength: our study used the manual muscle test with the Medical Research Council grading scale, while the aforementioned article used the manual muscle test, hand-held dynamometry (HHD), and the quick motor function test (QMFT), collectively. It reminds us that although the manual muscle test is convenient and simple, clinicians should also use more precise methods to fully assess the distribution of muscle weakness, such as the QMFT, HHD, and the computed tomography/magnetic resonance imaging muscle scans, especially for asymptomatic patients and those with minor muscle strength loss. Another hypothesis is that the distribution of muscle involvement differs among patients from distinct ethnic groups, but it requires further investigations.

We found out that patients who exhibited axial muscle weakness had earlier disease onset ages than those who did not, and a study about LOPD in 54 Dutch patients^[27] also revealed that the patients with scoliosis experienced their first complaints before 21 years of age, while the mean age for all 54 patients was 28.1 ± 14.3 years. The mechanism is not clear yet, but this discovery reminds us to pay more attention to the axial muscle strength for LOPD patients with early disease onset age.

Throughout our study, we tried to identify a genotype-phenotype correlation and found some supporting evidences. Patient one (P1) had a nonsense mutation and a missense mutation c.1201C>A which was predicted to be deleterious in two GAA alleles, while patient four (P4) had a nonsense mutation and a missense mutation c.796C>A predicted to be benign. The two compound heterozygous mutations of P1 were more deleterious than those of P4, which led to severer clinical symptoms. Compared to P4, P1 had earlier symptoms onset age (1 vs. 13 years), longer

disease duration (41 vs. 3 years), and severer muscle weakness in lower extremities (III/V vs. IV/V).

Unfortunately, we did not find the same correlation between the effects of *GAA* gene mutations and the severity of clinical presentations in other patients. There are several possible explanations. First, it is difficult to predict the combined effects of compound heterozygous mutations, since they can disturb GAA enzyme activity at various points of the protein synthesis processing and subsequently result in heterogeneous phenotypes.^[4] Second, certain modifying factors can affect the actions of GAA sequence variations. De Filippi *et al.*^[28] reported that polymorphisms of the angiotensin-converting enzyme (*ACE*) and alpha-actinin 3 (*ACTN3*) could modulate the clinical symptoms of Pompe patients. The DD genotype in *ACE* gene (absence or deletion of D in a 287-base-pair alu repeat within intron 16) and the XX genotype in *ACTN3* gene (R577X polymorphism with a C to T conversion at position 1747 in exon 16) were significantly associated with an earlier age of disease onset. Other genetic and nongenetic factors can also impact the clinical manifestations of LOPD patients. Third, the functions of prediction software programs are limited. They cannot predict the precise severity of the novel mutations. Fourth, the five cases in our study did not provide enough evidences to get exact conclusions. Thus, the correlations between genotype and phenotype of LOPD patients should be further investigated.

The three known mutations detected in our study are relatively common in Chinese LOPD patients. To the best of our knowledge, 18 articles about *GAA* gene mutations in Chinese LOPD patients have been published so far, nine in English and nine in Chinese.^[3,5-21] In these articles, a total of 107 patients from 98 families with 66 different mutations were reported, and 32 mutations were novel at the time of publication. The mutations are spread

throughout the 19 coding genes except for exons 3, 10, and 18. Exon 2, which contains a start codon, and exon 9 and 15, which are in the enzyme catalytic barrel, have the greatest number of mutations, in line with previous reports.^[4,29] Among the 66 mutations, 37 (56%) were reported only once, 22 (33%) were reported from two to five times, and seven (11%) were reported six or more times, correlating with previous reports.^[4] The most common mutations were pseudo-deficiency mutations c.1726G>A and mutation c.2238G>C, which accounted for 33 (16.8%) and 32 alleles (16.3%), respectively. Mutation c.2238G>C was prevalent in LOPD patients from the mainland of China, Taiwan, China, and Hong Kong, China, while mutation c.1726G>A was rarely detected in patients from the mainland of China. The Chinese founder mutation c.1935C>A and the combined mutation c.(752C>T; 761C>T) accounted for 25 (12.8%) and 16 alleles (8.2%), respectively, but these mutations were more prevalent among patients from Taiwan, China. As for the other two known mutations in our study, except for c.2238G>C (16.3%), c.1309C>T was detected in six alleles (3.1%), and c.2237G>A was detected in four alleles (2.0%).

To date, a few prevalent mutations in LOPD patients from other ethnic populations have been reported, but they are rarely detected in Chinese patients. Likewise, common mutations found in Chinese patients mentioned above are infrequent in other populations. For example, mutation c.-32-13T>G is the most frequent pathogenic mutation found in Caucasian individuals with an allele frequency of 42.3–47.4%,^[30-33] but it has been identified just two times in Chinese LOPD patients. Other common mutations, such as c.596G>A and c.668A>G (frequency per family 35.9%) in the Brazilian population,^[34] and c.2560C>T in the African-American population,^[35] have not been previously reported in Chinese LOPD patients. The above common mutations were not detected in our cohort of patients either. Combining with the fact that detection rate of novel mutations in Chinese patients is as high as 16.3% (32/196) and considering the findings described above, we believe that considerable genetic heterogeneity exists in LOPD patients from China.

The seven novel mutations found in our study expand the genetic spectrum of LOPD patients, and six of them were predicted to be deleterious. However, the pathogenicity of the seven mutations has not been verified by *in vivo* or *in vitro* experiments. Our future research will focus on the pathogenicity and severity rating of the novel mutations.

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Conflicts of interest

There are no conflicts of interest.

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5例中国晚发型Pompe病患者的7个GAA基因新发突变报道

摘要

背景: Pompe病是一种由酸性 α -1, 4-葡糖苷酶基因 (GAA基因) 突变引起的罕见的糖原累积病, 不同种族的Pompe病患者临床表型及基因型差异很大, 且其临床表型-基因型相关性尚不明确。本研究旨在报告5例伴有新发GAA基因突变的晚发型Pompe病患者的临床、病理及基因突变特点。

方法: 收集从1986年4月至2017年8月在我院诊断为糖原累积病的患者的临床及病理资料, 提取患者冰冻肌肉标本DNA进行二代测序和基因突变分析。

结果: 5例患者的发病年龄中位数为13岁, 病程中位数为5年。患者主要表现为进展性四肢近端肌肉及中轴肌无力, 其中1例患者出现了呼吸困难并需要呼吸机辅助呼吸。肌肉活检可见肌纤维出现大小及形态各异空泡样变性, PAS及ACP染色阳性。基因检测共发现到10个GAA基因突变, 其中7个为新发突变 (c.796C>A, c.1057C>T, c.1201C>A, c.1780C>T, c.1799G>C, c.2051C>A, c.2235dupG)。

结论: 7个GAA基因新发突变扩展了晚发型Pompe病患者基因表达谱, 并提示中国晚发型Pompe病患者有较强的遗传异质性。