Clinical and molecular genetic study of infantile-onset Pompe disease in Chinese patients: Identification of 6 novel mutations

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1. Introduction

Pompe disease (PD, OMIM #232300), also known as glycogen storage disease type II and acid maltase deficiency, is an autosomal recessive disorder of glycogen metabolism resulting from defects in the activity of lysosomal acid α-glucosidase (GAA; EC.3.2.1.20) (Hirschhorn and Reuser, 2001). The disease presents as a continuous clinical spectrum, although it can be broadly classified into infantile-onset and late-onset forms, according to the age of onset. Previous studies have made distinctions between “classic” and “atypical” forms of infantile-onset Pompe disease (Hirschhorn and Reuser, 2001; Slonim et al., 2000). The “classic” form applies to those infants who die within the first year of life, while the term “atypical” defines patients with milder myopathy who typically have respiratory failure between 12 to 18 months of age but can live longer with respiratory and nutritional support (Kishnani et al., 2006).

The incidence of Pompe disease varies in different ethnic groups and for the different clinical forms. The rapidly progressive infantile-onset form has estimated frequencies of 1:138,000 in the Caucasian population (Ausems et al., 1999), 1:50,000 in Taiwanese (Lin et al., 1987), and 1:31,000 in those of African ancestry (Kishnani et al., 2006). Yet, very few cases of infantile-onset Pompe disease have been reported in Mainland China.

Pompe disease is caused by mutations in the human gene GAA (OMIM #606800), which is located on chromosome 17q25.3 and is transcribed into three RNA isoforms encoding the same protein. The gene contains 20 exons (transcript variant 1, NM_000152), and the first exon is non-coding (Hoefsloot et al., 1988; Martiniuk et al., 1986).

The cDNA for GAA is greater than 3.6 kb in length with 2856 nucleotides of coding sequence and the resulting product is a protein of 952 amino acids (Martiniuk et al., 1990).

To date, 393 variations have been described in the GAA gene, 257 of which have been confirmed to be pathogenic (www.pompecenter.nl). Most mutations are extremely rare and limited to individual patients, but common mutations in some populations have been reported. In

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Abbreviations: GAA, acid alpha-glucosidase; PD, Pompe disease; PAS, periodic acid-Schiff; RT-PCR, reverse transcriptase–polymerase chain reaction; SIFT, Sorting Intolerant From Tolerant.

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Taiwanese populations, the c.1935C>A (p.Asp645Glu) mutation represents 36%–80% of mutations (Ko et al., 1999; Shieh et al., 1994); however, the frequency and distribution of GAA mutations in patients from the Chinese Mainland is unclear. Here, we present the clinical course of the disease and molecular findings in a population of Chinese Mainland patients with classic infantile-onset Pompe disease.

2. Material and methods

2.1. Ethics statement

This study was approved by the Medical Ethics Committee of Shanghai Children’s Medical Center and complied with the principles expressed in the Declaration of Helsinki. Written informed consent was given by the patients’ parents.

2.2. Patients and clinical data

Patients for this study were diagnosed with infantile-onset Pompe disease from 2005 to 2012 in Shanghai Children’s Medical Center. The diagnosis was based on clinical presentations and reduced GAA enzyme activity in leukocytes or dried blood spots. GAA enzyme activity was determined as previously described, with 4-methylumbelliferyl-α-D-glucopyranoside as the substrate and acarbose as an inhibitor of maltose glucoamylase using a fluorometric assay at pH 3.8 (Okumiya et al., 2006; Zhang et al., 2006). Confirmative diagnoses were conducted by GAA genotyping and/or by peripheral blood smear evaluation. Clinical data were retrospectively collected from medical records.

2.3. Peripheral blood smear evaluation

Four blood films were prepared from each examined patient. Two films were stained with Wright-Giemsa reagent, and the other 2 with periodic acid-Schiff (PAS) reagent. All blood films were prepared within 2 h of the sample being drawn. Staining and microscopic inspection of the blood films was carried out within 1–5 days (Hagemans et al., 2010).

2.4. GAA mutation analysis

Genomic DNA was extracted from peripheral blood leukocytes with the Genomic DNA Purification Kit (Qiagen, Germany). The coding region (exon2–exon20) and the intron/exon boundaries of GAA were amplified by PCR with previously reported primers (Ko et al., 1999). PCR products were analyzed using the Big-Dye Terminator Chemistry kit (Applied Biosystems, Foster City, CA, USA). Mutagen nomenclature follows guidelines set by the Human Genome Variation Society (www.hgvs.org/mutnomen/). NM_000152 was used as the reference sequence for the coding regions. Each novel missense mutation or previously reported variant with uncertain functional effect was screened in 60 normal individuals (120 alleles) using direct sequencing.

2.5. RT-PCR analysis of RNA splicing

The effect of novel splice site mutations was assessed by reverse transcriptase–polymerase chain reaction (RT-PCR) analysis. Peripheral blood mononuclear cells were obtained from the affected individuals. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed into cDNA using the SuperScript III First-Strand DNA Synthesis kit (Invitrogen, Carlsbad, CA, USA). PCR was performed with exon-specific primers. To examine the c.859-2 A>T mutation, a region encompassing exons 3–6 was amplified with primers GAA-3F: 5'-ATCAAGATCCACCTAAAGGG-3' (exon 3, nts 547–567) and GAA-6R: 5'-AGGAAGATGTGACATCCAGGT-3' (exon 6, nts 1028–1006). The effect of the c.1551 + 2T>G mutation was studied by amplification of exons 9–12 with primers GAA-9F: 5'-GGTTTTCATCACAACGAGAC-3' (exon 9, nts 1395–1415) and GAA-12R: 5'-AGGTGTTAAGTGTGAGAGAAAC-3' (exon 12, nts 1712–1689). The PCR products were analyzed by electrophoresis on 2% ethidium bromide-stained agarose gels, and cloned into the pMD19-T vector for subsequent sequence analysis.

2.6. In silico analysis of missense mutation

The possible pathogenic nature of each novel missense mutation or previously reported variant with unknown functional effect was assessed using two bioinformatics tools, Sorting Intolerant from Tolerant (SIFT) (Ng and Henikoff, 2003) (through SNP nexus (Chelala et al., 2009); http://snp-nexus.org/index.html) and Polymorphism Phenotyping-2 (Adzhubei et al., 2010) (PolyPhen-2; http://genetics.bwh.harvard.edu/pph2/).

3. Results

3.1. Patients and clinical data

Eighteen unrelated patients were included in this study. The patients came from different parts of Mainland China, with 15 of them from southern China (south of the Yangtze River) and the other 3 from northern China (north of the Yangtze River). Four patients had a family history of Pompe disease. All the patients presented with hypotonia and cardiomyopathy prior to one year of age. Feeding difficulties and/or failure to thrive were the first symptoms in 10 (55.6%) patients. Motor problems (such as muscular weakness, motor retardation, and paucity of movements) were the first symptoms in 8 (44.4%) patients. Respiratory problems (such as recurrent airway infections and respiratory distress) were the first symptoms in 13 (72.2%) patients. Cardiac problems (such as cardiac failure and cardiomegaly) were noticed as the first symptoms in 14 (77.8%) patients. The median age at symptom onset was 3.6 months (range: 1.7–6.8 months), and median age at diagnosis was 6.3 months (range: 2.5–9.3 months). All but 1 patient (94.4%) died during follow-up, and the median age at death was 8.2 months (range: 4.7–18.7 months) (Table 1, Fig. 1). The only surviving patient was 5.5 months old at the time of the last follow-up.

3.2. Peripheral blood smear evaluation

A peripheral blood smear evaluation was performed in 4 patients. In all 4, blood films showed the presence of well-demarcated, small, cytoplasmic vacuoles in the lymphocytes. Cytochemistry revealed granular positivity of lymphocytes subjected to the PAS reaction (Fig. 2).

3.3. Molecular characterization

We performed molecular analysis of the GAA gene in 18 patients with infantile-onset Pompe disease. In 14 (77.8%) patients, we identified mutations in both alleles, but in the other 4 (22.2%) patients, only 1 mutation was detected (Table 2). The mutations we observed were spread over the whole region of the GAA gene (Fig. 3). Of the 20 different mutations that we identified, 6 have not been previously reported. Among the novel mutations, 3 of them lead to premature stops in protein synthesis: c.1356delC (p.Ser454AlafsX23), c.378G>T (p.Tyr609X), and c.1827C>G (p.Tyr609X). Two splice site mutations (c.859-2 A>T, c.1551 + 2T>G) appear to fully prevent correct splicing. Furthermore, 1 novel missense mutation c.1465G>T (p.Asp489Tyr) was identified in this study.

To investigate the effect of the variant (c.859-2 A>T) on splicing of the GAA gene, the region encompassing exons 3–6 was amplified by RT-PCR. The amplicons were analyzed by agarose gel electrophoresis, and two different PCR fragments were observed for the affected individual. Further cloning and sequencing analysis showed that the 482 bp
fragment corresponded to normally spliced mRNA, whereas the 559 bp fragment corresponded to an mRNA retaining the full sequence of intron 4 (Fig. 4). Retention of intron 4 would shift the reading frame, fragment corresponded to in-frame skipping of the entire exon 10 (Fig. 5).

Furthermore, we performed an in silico analysis of the novel missense mutation c.1465C>T (p.Asp489Tyr). The aspartic acid at position 489 is highly conserved among 45 compared vertebrates, and it occurs within a relatively conserved region, which suggests that this amino acid plays an essential role in GAA enzyme activity. SIFT and Polyphen predicted the alteration p.Asp489Tyr to be damaging. Among previously reported mutations, c.1832G>A (p.Gly611Asp), which is a variant with unknown functional effect, was also identified in this study. SIFT and Polyphen analysis suggested that the alteration of p.Gly611Asp could lead to dysfunction of GAA (damaging with high confidence in both SIFT and Polyphen prediction). Moreover, neither variant c.1465C>T nor c.1832G>A was detected among 120 alleles of normal controls.

The most common mutation in this study was the c.1935C>A (p.Asp645Glu) mutation, which was found in 9 out of 36 alleles (25%). Seven patients were heterozygous, and 1 patient was homozygous for the mutation. Of the 8 patients, only 1 patient originated from the northern Chinese population, whereas the other 7 patients originated from the southern Chinese population. Furthermore, we observed that all the alleles carrying the c.1935C>A mutation were linked to a specific haplotype (c.324T>C, c.1203G>A, c.1726G>A, c.2065G>A, and c.2338G>A).

4. Discussion

Pompe disease is an autosomal recessive disorder caused by the deficiency of functional GAA enzyme, which is responsible for the degradation of glycogen to glucose in the acidic environment of the lysosome. Loss of GAA enzyme activity results in lysosomal glycogen accumulation and, consequently, leads to progressive tissue damage, with the cardiac, skeletal, and smooth muscle cells being the most markedly affected. Classic infantile-onset Pompe disease is the most aggressive and life-threatening form of the disease. In this study, all patients presented with a typical clinical course of the infantile-onset form, which was characterized by hypertrophic cardiomyopathy and profound generalized weakness presenting in the first few months of life, with rapid progression and death usually occurring by one year of age.

Van den Hout et al. (van den Hout et al., 2003) compared the natural course of classic infantile Pompe disease among 20 original Dutch cases and 133 previously published cases. They observed that the median age...
at symptom onset was 1.6 months in both groups; the median age at diagnosis (and death) was 5.3 (7.7) and 4.5 (6.0) months in the Dutch cohort and in the literature cases, respectively. The natural course of infantile Pompe disease has also been reported in a large retrospective chart review of 188 cases, which included patients with atypical as well as classic infantile-onset form (Kishnani et al., 2006). In the large review study, the median age at symptom onset was 4 months, diagnosis at 4.7 months, and death occurred at 8.7 months. In our study, the median age at symptom onset was 3.6 months and diagnosis at 6.3 months. All but 1 patient died during follow-up, and the median age at death was 8.2 months. Our data correlates with the similar age of death reported by the other two studies mentioned, but the age at diagnosis was considerably older than that in the previous studies. Lack of awareness and qualified diagnostic tests might be the reason for the late diagnosis in Mainland China.

In this study, we identified 3 novel allelic variants predicted to encode truncated forms of GAA. The substitution c.378G > A (p.Trp126X) is the second mutation identified in this codon. The c.377G > A (p.Trp126X) mutation at the same codon has been observed commonly in the Argentinean Pompe patients of Italian origin, in both infantile-onset and late-onset cases (Oba-Shinjo et al., 2009; Palmer et al., 2007). The substitution c.1827C > G (p.Tyr609X) is the third mutation identified in this codon. Previously, c.1826dupA (p.Tyr609X) was detected in a Dutch patient with infantile-onset type, whereas c.1827del (p.Tyr609X) was detected in an Australian patient with late-onset type. Both of the 2 nonsense mutations were reported as pathogenic sequence variations (Hermans et al., 2004). The single base deletion c.1356delC causes a frameshift after codon 454, resulting in a premature terminator that is 23 codons downstream. The truncated protein likely does not have a functional catalytic site, normally located between codons 516 and 520 of the GAA gene (Hermans et al., 1991). Marked reduction in enzyme activity and severe phenotypic effects can be expected.

Two novel splice-site mutations were observed in this study. The c.859-2A > T mutation in IVS4 disrupts the conserved splice acceptor site at the junction of exon 5. Patient PD7 carrying this mutation, along with the previously reported mutation c.2662C > T, presented with severe infantile disease, which suggested that the mutation in IVS4 abrogates normal splicing. Further analysis on mRNA of GAA gene confirmed the presence of 2 aberrant transcripts in the affected individual: one showed full retention of IVS4 and the other showed partial deletion of exon 5, which might be due to the activation of a cryptic splice site. The latter transcript could be identified only by DNA sequence analysis of the cloned RT-PCR product, suggesting a very low expression level of the transcript. However, both of the aberrant variants were predicted to result in an early truncation of the GAA protein upstream of the functional catalytic site. The c.1551 + 2T > G mutation in IVS10 disrupts the conserved splice donor site at the junction of exon 11. Patient PD8 with this mutation, along with the previously reported mutation c.1935C > A, presented with severe infantile disease, suggesting that the mutation in IVS10 could lead to aberrant splicing. Further analysis on mRNA demonstrated that the c.1551 + 2T > G mutation disrupted the splice donor site and caused an in-frame deletion of exon 10. The abnormal splicing would result in the exclusion of a coding region, which includes part of the enzyme catalytic site as well as disruption of an essential region around the catalytic site (Huie et al., 1994).

Furthermore, we identified a novel missense mutation c.1465G > T (Asp489Tyr). Interestingly, a G > A substitution at nucleotide position 1465 has been observed in an Italian late-onset patient, and this substitution produces a p.Asp489Asn mutation and consequently abolishes the GAA activity (Montalvo et al., 2006). The disease-causing effect of the c.1465G > T (Asp489Tyr) mutation was not clear, since few associated studies have been reported. Yet, the absence of these mutations on the 120 alleles of normal controls, with the conservation of the affected amino acids among 45 vertebrate genomes, makes it very likely that the mutations can be directly correlated to disease state. Nevertheless, the effect of the novel mutation should be assessed by functional studies.

Mutation c.1935C > A (p.Asp645Glu) is of interest among the previously reported mutations because it has been widely detected in

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**Table 2**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Nucleotide change</th>
<th>Amino acid alteration</th>
<th>Nucleotide change</th>
<th>Amino acid alteration</th>
<th>Family history</th>
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<td>PD2</td>
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<td>unknown</td>
<td>Y</td>
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<tr>
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<td>c.2186delC</td>
<td>p.Thr729fsX8</td>
<td>unknown</td>
<td>unknown</td>
<td>Y</td>
</tr>
<tr>
<td>PD6</td>
<td>c.784G &gt; A</td>
<td>p.Glu262lys</td>
<td>c.1356delC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>p.Ser454AlafsX23</td>
<td>N</td>
</tr>
<tr>
<td>PD7</td>
<td>c.2662G &gt; T</td>
<td>p.Glu888X</td>
<td>c.859-2A &gt; T</td>
<td>Splice site</td>
<td>N</td>
</tr>
<tr>
<td>PD8</td>
<td>c.1935C &gt; A</td>
<td>p.Asp645Glu</td>
<td>c.1551 + 2T &gt; G</td>
<td>Splice site</td>
<td>N</td>
</tr>
<tr>
<td>PD10</td>
<td>c.118C &gt; T</td>
<td>p.Arg40X</td>
<td>c.2815_2816del</td>
<td>p.Val939fsX78</td>
<td>N</td>
</tr>
<tr>
<td>PD12</td>
<td>c.1827C &gt; G</td>
<td>p.Tyr609X</td>
<td>c.1465G &gt; T</td>
<td>p.Asp489Tyr</td>
<td>Y</td>
</tr>
<tr>
<td>PD13</td>
<td>c.2662G &gt; T</td>
<td>p.Glu888X</td>
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<tr>
<td>PD14</td>
<td>c.2662G &gt; T</td>
<td>p.Glu888X</td>
<td>c.2432delT</td>
<td>p.Leu811fsX36</td>
<td>N</td>
</tr>
</tbody>
</table>

<sup>a</sup> Novel GAA mutations are indicated in bold.

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![Figure 2](https://example.com/figure2.png)

**Figure 2.** Photomicrographs of blood films from a patient with classic infantile-onset Pompe disease. Left: Wright-Giemsa-stained blood film of a patient showing a lymphocyte with several distinct vacuoles; Right: PAS-stained blood film of a patient showing a lymphocyte with PAS-positive inclusions.
Fig. 3. Mutation spectrum in 18 Chinese patients with infantile-onset Pompe disease. Previously described mutations are shown in black, and new mutations are shown in red (red: UTR; blue: introns; green: exons).

Fig. 4. Aberrant splicing induced by the c.859-2 A → T mutation. Panel a. Schematic representation of the 2 splicing patterns generated by this variant. MT1 retains the entire intron 4, and MT2 carries the deletion of the first 86 bp of exon 5. Panel b. Gel electrophoresis of RT-PCR products. Lane 1 and lane 2 represent the heterozygous mutation carrier and normal control, respectively. Lane M, DL2000 DNA marker. Panel c. Partial nucleotide sequence of 2 cloned mutant transcripts derived from GAA cDNA of the mutation carrier. The mutant transcript MT1 shows retention of entire intron 4, and MT2 shows a deletion of the first 86 bp of exon 5.
African–American, Japanese, Taiwanese, and Thai patients. However, the reports of frequency are unclear for different regions. For instance, one study reports that mutation c.1935C\textsuperscript{N}A accounts for 80% of the mutant alleles in the Taiwanese patients (Shieh et al., 1994), whereas another study reports the same mutation only accounts for 36% of the mutant alleles in the same population (Ko et al., 1999). More interesting is the demographic distribution of the patients in these two studies. All of the cases were found to originate from the southern Chinese population and a group of aborigines in Taiwan, whereas none was found to be of northern Chinese origin. Amarinthnukrowh et al. (Amarinthnukrowh et al., 2010) reported that mutation c.1935C\textsuperscript{N}A accounted for 80% of the mutant alleles in Thai patients and is the most common mutation in the Thai population. Although this mutation was observed with the most frequency of any mutation in the present study, it only accounted for 25% of alleles. Among the 8 patients with the mutation, we found 1 patient originated from the northern Chinese population, and the other 7 patients originated from the southern Chinese population. Furthermore, we observed that the c.1935C\textsuperscript{A} mutation was linked to a specific haplotype (c.324T\textsuperscript{C}A, c.1203G\textsuperscript{A}, c.1726G\textsuperscript{A}, c.2065G\textsuperscript{A}, c.2338GA), which is consistent with those found in the infants from the Taiwanese population. Taken together with previous studies, our findings provide evidence for a common origin of the c.1935C\textsuperscript{A} (p.Asp645Glu) mutation (Shieh and Lin, 1998). The c.1726G\textsuperscript{A} (p.Gly576Ser) variant in cis with c.2065G\textsuperscript{A} (p.Glu689Lys), also known as the c.[1726A; 2065A] pseudodeficiency allele, causes low GAA activity in normal individuals and is relatively common in Asian populations (Kroos et al., 2008). The mutation c.1935C\textsuperscript{A} has been consistently observed in this haplotype context and is associated with infantile-onset Pompe disease, which was also found in our current study. It has been suggested that the pseudodeficiency background might be required for c.1935C\textsuperscript{A} to present as a severe mutation (Labrousse et al., 2010).

The missense mutation c.1832G\textsuperscript{A} (p.Gly611Asp), a previously reported variant with unknown functional effect (Kroos et al., 2012), was also identified in this study. Patient PD18, carrying the mutation in combination with the previously reported mutation c.1843G\textsuperscript{A}, presented clinical symptoms at 1.7 months. The absence of other identifiable mutations in the patient, along with the predicted damaging effect of the mutation based on PolyPhen and SIFT, suggests that the change likely has functional significance. In addition, we did not observe the variant allele upon sequence analysis of 120 normal Chinese GAA alleles, which indicated that it is not a common polymorphism in the general population.

In conclusion, our data offer the first comprehensive analysis on the clinical course and the mutational spectrum of infantile-onset Pompe disease in Mainland China. Our study resulted in the discovery of 6 novel mutations, and the mutational spectrum indicates that the c.1935C\textsuperscript{A} substitution is the most common mutation in Mainland Chinese patients with infantile-onset Pompe disease.

Conflict of interest

All the authors have no conflict of interest.
Acknowledgments

The research was supported by grants from the Innovation Program of Shanghai Municipal Education Commission (no. 12YZ035), National Natural Science Foundation of China (no. 81170151, 81170811 and 30973216), Shanghai School of National Health Bureau (no. 12ZZ114), Shanghai Municipal Technology and R&D Program (no. 2012BA098B04). The authors wish to thank Dr. Chong Li (Chinese National Human Genome Center at Shanghai) for his critical review of the manuscript. The authors wish to thank Ms. Yilei Qian for her excellent technical assistance.

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