



Development of a feasible assay for the detection of GAA mutations in patients with Pompe disease



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ABSTRACT

Background: Pompe disease is an inherited autosomal recessive deficiency of acid α -glucosidase (GAA) and is due to pathogenic sequence variants in the corresponding GAA gene. While the analysis of enzyme activity remains the diagnostic test of choice for individuals with Pompe disease, mutation analysis remains for establishing a definitive diagnosis.

Methods: High resolution melting (HRM) analysis was performed to screen GAA mutations. Genomic DNA was extracted from peripheral blood samples of the two patients with Pompe disease and 250 normal controls. Exons 2 through 20 of the GAA gene were screened by the HRM analysis. The results were subsequently confirmed by direct sequencing.

Results: This assay proved to be feasible in detecting seven known (c.2T>C, c.1726G>A, c.1845G>A, c.1935C>A, c.1958C>A, c.2238G>C, and c.2815_2816del) GAA mutations. Each mutation could be readily and accurately identified in the difference plot curves. We estimated the carrier frequency of the most common mutation, c.1935G>A (p.D645E), in the Taiwanese population to be 0.2%.

Conclusions: In clinical practice, we suggest that HRM analysis is assumed as a fast and reliable method for screening GAA gene mutations especially the most common mutations which are responsible for Pompe disease among the Taiwanese populations.

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1. Introduction

Pompe disease (glycogen storage disease type II, acid maltase deficiency, OMIM 232300) is an autosomal recessive progressive muscular disorder caused by the deficiency of acid α -glucosidase (GAA; EC.3.2.10.20) that results in impaired lysosomal degradation and the

Abbreviations: GAA, acid α -glucosidase; HRM, high resolution melting; DBS, dried blood spot; PCR, polymerase chain reaction; WCN, weighted contact number; DHPLC, denaturing high performance liquid chromatography.

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accumulation of glycogen. The lysosomal glycogen accumulated in many tissues such as skeletal, cardiac, and smooth muscles [1]. Pompe disease is classified into infantile, juvenile, and adult types based on clinical presentation and the onset of the disorder. The clinical manifestations of the infantile form are cardiomyopathy with cardiomegaly and rapidly progressive myopathy [1]. Late-onset Pompe disease is the result of a partial deficiency of GAA. The primary symptom is muscle weakness progressing to respiratory weakness. They may develop respiratory failure at a later stage during the course of the disease [2,3]. In general, GAA activity correlates with the age at onset and severity of disease. Infantile patients have less than 1% GAA activity, and late-onset patients have 2–40% of normal GAA activity in cultured skin fibroblasts [4]. The estimated frequency of Pompe disease in southern China and Taiwan newborns is 1 in 20,000–40,000 [5]. The combined incidence of all forms of Pompe disease is approximately 1:40,000 [6].

Measurement of GAA activity in skin fibroblasts is the current gold standard for the diagnosis of Pompe disease. Recently, isolation of GAA

from the dried blood spot (DBS) extracts has been developed for GAA activity assay. These novel DBS methods are not only suitable for newborn screening of Pompe disease, but also provide a quick and noninvasive diagnostic test for Pompe disease [1]. While enzyme activity analysis remains the diagnostic test of choice for individuals with Pompe disease, mutation analysis remains important. It is particularly useful in identifying carriers when a familial mutation is known. In addition, there are also some common mutations with useful genotype–phenotype correlations [3,7]. On the other hand, the availability of enzyme replacement therapy has increased the demand for rapid and more sophisticated diagnosis at both enzymatic and molecular level [8].

The human structural gene encoding GAA is located at chromosome 17q25.2–q25.3 and contains 20 exons, the first of which is non-coding [9]. The GAA cDNA is over 3.6 kb in length and with 2856 nucleotides of coding sequence. The resulting product is a protein of 952 amino acids [10,11]. The start codon begins at position 33 of exon 2 [9]. Mutations are randomly spread over the whole gene and some mutations appear with considerable frequency in distinct ethnic groups [12].

High-resolution melting (HRM) analysis is a mutation-scanning technology and offers considerable time and cost savings compared with screening methods. HRM analysis has recently been tested in a variety of clinical mutation-scanning and genotyping applications and is shown to be sensitive, cost-effective, and economical. Unlike other scanning method (such as sequencing), it is time-consuming and labor intensive. HRM analysis offers a faster and more convenient closed-tube method of assessing the presence of mutations and gives a result that can be further investigated if it is of interest. Besides, the closed-tube system reduces the risk of contamination. van der Stoep N et al. [13] compared HRM analysis with sequence analysis, which is the current gold standard for mutation detection in most diagnostic laboratories. They reported that HRM analysis is a highly sensitive method with a sensitivity of 100% in detecting all heterozygous mutations. Similarly, a meta-analysis [14] indicated that HRM analysis is a highly sensitive, simple and low-cost test to detect human disease-associated mutations, especially for samples with mutations of low incidence. Recently, our review article [15] also discussed about the effectiveness and limitations of HRM analysis in the diagnosis of genetic disorders including autosomal recessive, dominant, and X-linked disorders.

Identification of mutations in the GAA coding sequence is advantageous that it can provide a noninvasive method for the confirmation of the clinical and biochemical diagnosis. In addition, it is useful in identifying rare or novel mutations and facilitates genotype–phenotype correlations. Therefore, this present study aimed to assess the value of the HRM analysis using real-time polymerase chain reaction (PCR) (LightCycler® 480; Roche Applied Science) for scanning GAA gene mutations in patients with Pompe disease.

2. Materials and methods

2.1. Diagnosis of patients

Confirmatory diagnosis included both biochemical and molecular tests. GAA activity in DBS was measured using artificial substrate 4-methylumbelliferyl- α -D-glucopyrosidase (Fluka Chemical Corp, Ronkonkoma, NY, USA) and the nature substrate glycogen. Genomic DNA was isolated using the proteinase K and QIAamp® mini DNA extraction kit (QIAGEN) according to the manufacturer's protocol. The quality and quantity of the extracted DNA samples were measured using the Nano-200 Nucleic Acid Analyzer (MEDCLUB, Tainan, Taiwan). This study was approved by the Institutional Review Board (IRB) of Kaohsiung Medical University Hospital (KMUHIRB-20130027).

2.2. Optimization of HRM curve analysis

Seven DNA samples with previously identified GAA mutations were used for the optimization and validation of HRM. Optimized HRM

conditions were applied for further screening of GAA mutations in two additional Pompe patients from our medical center. Meanwhile, we also recruited 250 healthy subjects in order to estimate the allele frequency of the most common GAA gene mutation, c.1935C>A (p.D645E).

2.3. Assay design and PCR conditions

Good amplicon design is essential to obtain a robust and reproducible HRM analysis. The difference between wild-type and heterozygote curves became smaller and more difficult to differentiate when the product length was increased [16]. Besides, extra care is needed to design PCR conditions to avoid primer dimers and non-specific amplification in HRM analysis. In this study, all 25 pairs of primers for HRM analysis were newly designed using Primer3 software (Supplementary data 1). For exon 2 and exon 4, six sets and two sets of primers were used to amplify the exons in overlapping segments, respectively. All synthesized primers were of standard quality in molecular biology quality (Protech Technology Enterprise Co., Ltd, Taiwan).

2.4. The HRM technique

PCR reactions were carried out in duplicate in 10 μ L final volume using the LightCycler® 480 High-Resolution Melting Master (reference 04909631001, Roche Diagnostics) 1 \times buffer, containing Taq polymerase, nucleotides and the dye ResoLight, and 50 ng DNA. The primers and MgCl₂ were used at a concentration of 0.25 μ M and 2.5 mM, respectively for detecting the GAA gene mutations. The HRM assays were conducted using the LightCycler® 480 Instrument (Roche Diagnostics) provided with the software LightCycler® 480 Gene-Scanning Software Version 1.5 (Roche Diagnostics). The PCR program required a SYBR Green I filter (533 nm), and it consisted of an initial denaturation–activation step at 95 °C for 10 min, followed by a 45-cycle program (denaturation at 95 °C for 15 s, annealing at 58 °C or 60 °C for 15 s and elongation at 72 °C for 15 s with the reading of the fluorescence; acquisition mode: single). The melting program included three steps: (a) denaturalization at 95 °C for 1 min, (b) renaturation at 40 °C for 1 min, and (c) subsequent melting that consists of a continuous fluorescent reading from 60 to 90 °C at the rate of 25 acquisitions per degree centigrade. The difference plot curves of the duplicate for each DNA sample must be reproducible both in shape and peak height.

2.5. Sequencing of GAA exons 2–20

Exons 2 through 20 of the GAA gene were amplified by PCR as previously described with some modified reaction conditions [17]. Each locus was amplified in a 20 μ L volume including buffer [60 mM Tris–SO₄ (pH 8.9), 18 mM (NH₄)₂SO₄, 2 mM MgSO₄], 0.2 mM dNTP, 0.2 μ M forward PCR primer, 0.2 μ M reverse PCR primer, 50–120 ng genomic DNA and 1 U of AccuPrime Taq DNA polymerase (Invitrogen Canada Inc., Ont.). The DNA was amplified using the following amplification conditions: 94 °C for 3 min; 35 cycles of 92 °C for 15 s, 50 °C for 15 s, and 68 °C for 1 min. After PCR, the samples were purified using the Geneaid Gel/PCR DNA fragments extraction kit. The sequence reaction was performed in a final volume of 10 μ L, comprising 1 μ L of the purified PCR product, 2.5 μ M of each PCR primer and 2 μ L of ABI PRISM terminator cycle sequencing kit v3.1 (Applied Biosystems). The sequencing program is a 25-cycle PCR program (denaturation at 96 °C for 10 s; annealing at 50 °C for 5 s and elongation at 60 °C for 4 min). The sequence detection was performed in the ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

2.6. Weighted contact number (WCN) and the sequence conservation profiles

Protein packing is described using weighted contact number [18]. The weighted contact number (WCN) of an atom is the sum of the inverse square of the distances between it and other atoms. The sequence-specific

conservation scores were computed following the protocol of CONSURF [19], based on the phylogeny of the sequences. The conservation scores were normalized to the corresponding z-scores in such a way that the average conservation score is zero and the standard deviation is one. The series of the conservation scores of a sequence is referred to as its conservation profile. Notably, in the conservation profile of a protein, the residue of a lower conservation score is more conserved than that of a higher conservation score [20].

2.7. Structure prediction of human GAA protein

Three-dimensional (3D) structures of the human GAA were predicted using the (PS)² server [21]. (PS)² automatically uses an effective consensus strategy. The above mentioned strategy combines structural- and profile-based [22] comparison methods for both template selection and target-template alignment. (PS)² server selected the Human intestinal maltase-glucoamylase (PDB entry: 2QMJ) [23] as the model template.

3. Results

3.1. Diagnosis and clinical manifestations in patients

3.1.1. Patient 1

A 5 year-old boy was presented with a gross motor developmental delay since infantile. He started to head control when he was 5 months old. Then, he could sit alone at the age of 9 months and be able to walk independently at the age of 1 year and 9 months. He could not jump high even when he was 3-years-old. He also had difficulty in climbing up

stairs and running. At his first visit to our clinic at the age of 3 years, the patient showed positive Gowers' sign and deep tendon reflexes in his lower extremities were absent. Creatine kinase levels were mildly elevated. Muscle biopsy revealed intracytoplasmic huge autophagic vacuoles with high acid phosphatase activity. Glycogen storage disease type II was thus suspected; DBS were collected for GAA activity assay and it was confirmed by Pompe disease due to the deficient activity of acid α -glucosidase (GAA) in net GAA (with inhibitor), 0.29 $\mu\text{mol/L/h}$ (reference range: $12.22 \pm 5.88 \mu\text{mol/L/h}$). Mutational analysis showed that he harbored compound heterozygous mutations (p.R594H/p.D645E) (Fig. 1). His muscle biopsy results were shown in Fig. S1(C) & (D).

3.1.2. Patient 2

An 11 year-old girl presented with slowly progressive lower extremity weakness since the age of 10 years. She had mild difficulty in climbing upstairs and could not do sit-ups at all. On her first visit to our clinic, marked abdominal and tongue weakness were observed. Proximal muscle weakness was relatively mild but Gowers' sign was positive. Creatine kinase levels were mildly increased to 300 IU/L and muscle biopsy was thus performed. Cytoplasmic bodies with high acid phosphatase activity were found in some muscle fibers. Late-onset glycogen storage disease type II was therefore highly suspected. DBS were collected for GAA activity assay. Pompe disease was confirmed by deficient activity of acid α -glucosidase (GAA) in net GAA (with inhibitor), 0.26 $\mu\text{mol/L/h}$ (reference range: $12.22 \pm 5.88 \mu\text{mol/L/h}$). Mutational analysis showed that she harbored compound heterozygous mutations (p.R190H/p.D645E) (Fig. 2). Her muscle biopsy results were shown in Fig. S1(A) & (B).

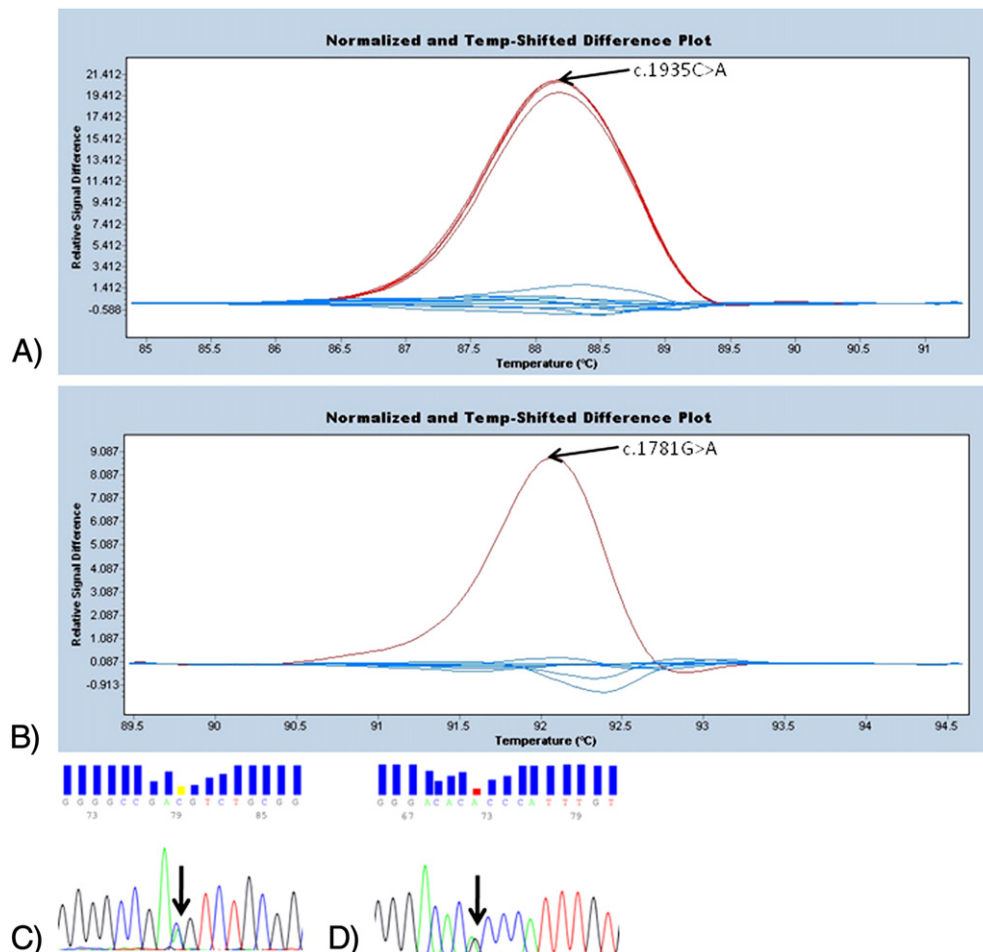


Fig. 1. The melting profiles of the GAA mutations in patient 1. (A) Normalized and temperature-shifted difference plots, the melting profile of c.1935C>A; (B) normalized plots, and normalized and temperature-shifted difference plots, the melting profile of c.569G>A; electropherogram of mutation: (C) c.1935C>A; Electropherogram of mutation: (D) c.569G>A.

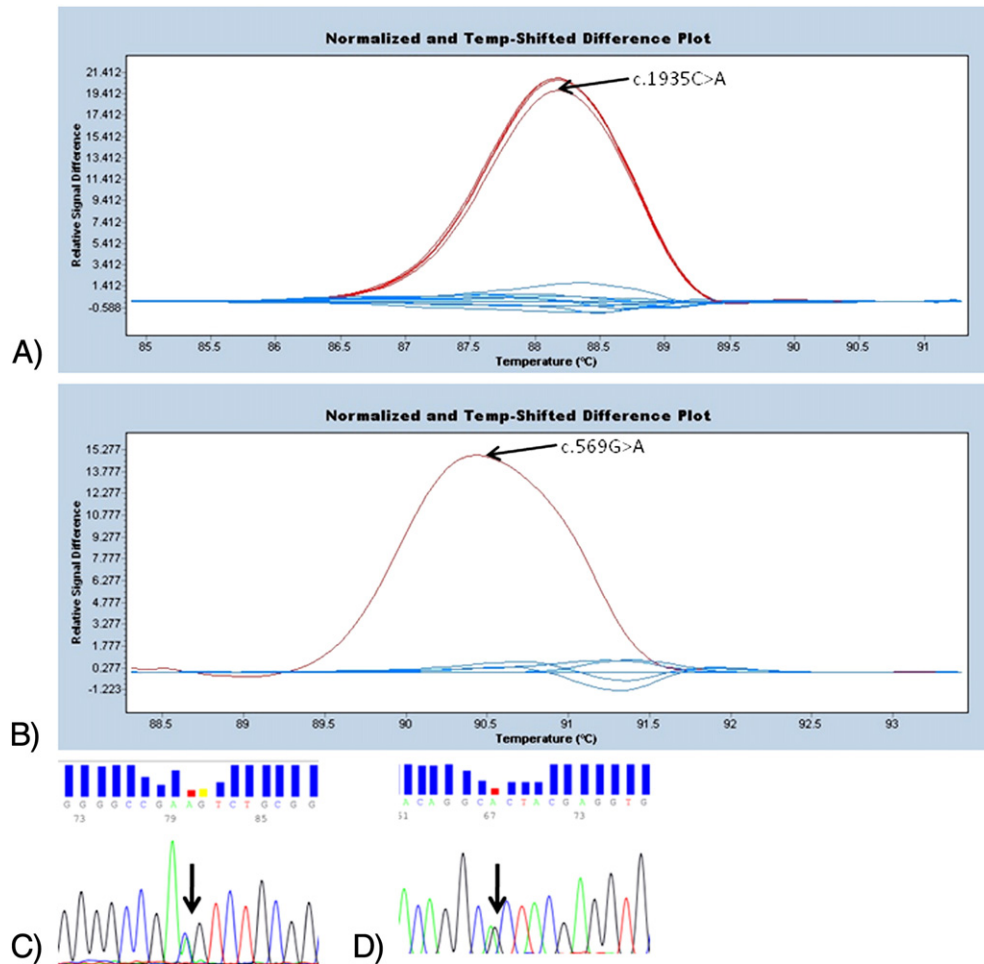


Fig. 2. The melting profiles of the GAA mutations in patient 2. (A) Normalized and temperature-shifted difference plots, the melting profile of c.1935C>A; (B) normalized plots, and normalized and temperature-shifted difference plots, the melting profile of c.1781G>A; electropherogram of mutation: (C) c.1935C>A; electropherogram of mutation: (D) c.1781G>A.

3.2. Optimization of HRM curve analysis

Seven known GAA mutations (c.2T>C, c.1726G>A, c.1845G>A, c.1935C>A, c.1958C>A, c.2238G>C, and c.2815_2816del) were used to assess the sensitivity of the HRM method for mutation scanning. Complete GAA mutational screening required investigation of exon 2 through exon 20. Twenty five PCR amplicons (169–377 bp) were evaluated with an average of 214 bp to cover exon 2 through exon 20 using LightCycler® system.

The normalized and temperature-shifted difference plots, the melting profile of GAA mutations c.2T>C, c.1845G>A, and c.2815_2816del are shown in Figs. S1, S2, and S3, respectively. The results were confirmed by direct sequencing. Fig. 3 shows that c.1726G>A (heterozygous, homozygous, and wild-type) are clearly described in the normalized temperature-shifted difference plots. Fig. 4 shows that c.1935C>A (heterozygous, homozygous, and wild-type) are clearly described in the normalized temperature-shifted difference plots. Fig. 5 shows that c.2238G>A (heterozygous, homozygous, and wild-type) are clearly described in the normalized temperature-shifted difference plots. All results were confirmed by direct sequencing. Intriguingly, we identified a non-pathogenic mutation among the Taiwanese population, c.1920T>G (p.P640P) [24] in normal populations (Fig. S4).

3.3. Allele frequency of the most common mutation, c.1935C>A (p.D645E), among the Taiwanese population

In the current study, we recruited 250 healthy Taiwanese subjects. We screened the subjects for the most common mutation,

c.1935C>A (p.D645E), using HRM analysis and computed the allele frequency for this mutant site. The results were confirmed by direct sequencing. We found that only one subject harbors a heterozygous mutation, c.1935C>A (p.D645E). The estimated allele frequency was 0.002 for c.1935C>A (p.D645E).

3.4. Structure packing density and sequence conservation analyses of the GAA protein

The protein packing density is described using weighted contact number (WCN) [25]. The larger the WCN of a residue, the more packed its environment. The reciprocal of the WCN (rWCN) profile is used to be compared with the sequence conservation profile. The conservation scores of a protein were calculated using ConSurf [19]. These scores are a relative measure of evolutionary conservation at each sequence site of target chain. The lowest score represents the most conserved position in a protein. Qualitatively, the residues of larger WCNs (or in more packed environments) are more conserved than those of smaller WCNs (or in less packed environments). Indeed, Fig. 6 demonstrates that rWCN profile (dotted line) and the conservation profile (solid line) overlap extremely well in the GAA protein. The recent study shows that the catalytic site residues tend to be located in regions of high packing density [26]. Our results demonstrate that these mutations (p.R190H, p.G576S, p.R594H, p.D645E, and p.W746C) are conserved residues and also located in the more packed environments. Therefore, it is reasonable to assume that these residues are highly associated with the functionality of the GAA protein.

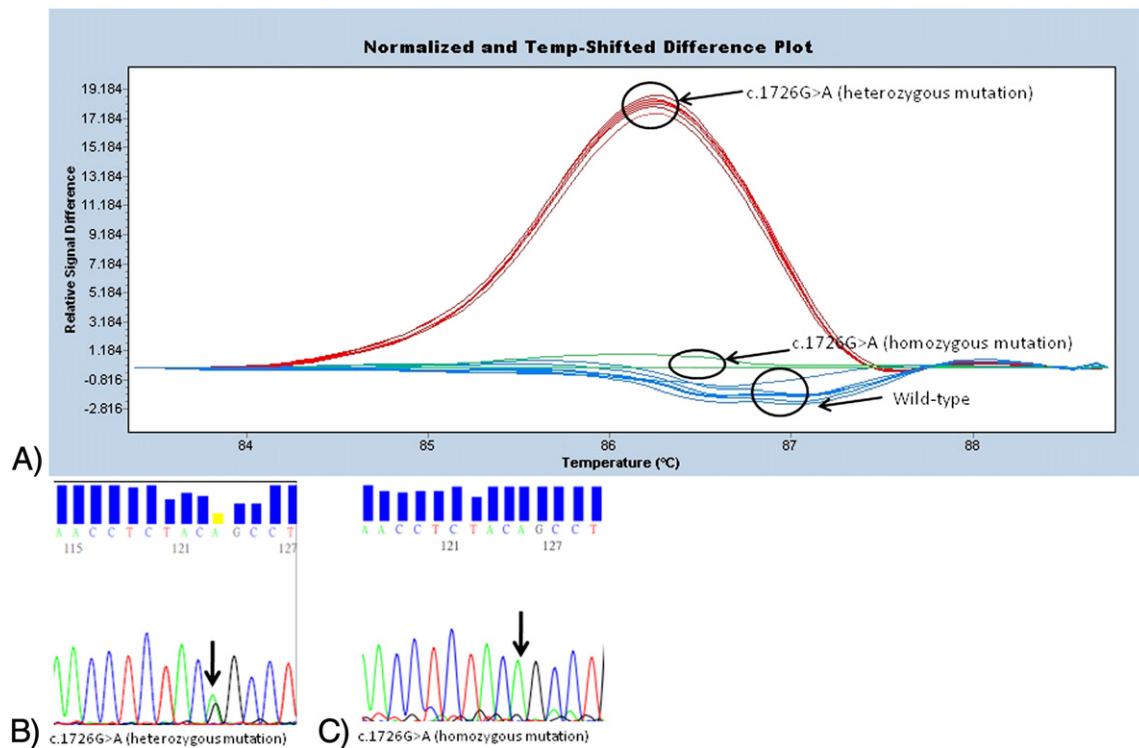


Fig. 3. Optimization of HRM curve analysis specific for c.1726G>A. (A) Normalized and temperature-shifted difference plots, the melting profile of c.1726G>A (homozygous mutation, heterozygous mutation, and wild-type); electropherogram of mutation: (B) c.1726G>A (heterozygous mutation); (C) c.1726G>A (heterozygous mutation).

4. Discussion

In this study, we have successfully established a diagnostic strategy by HRM analysis for identifying seven known (c.2T>C, c.1726G>A, c.1845G>A, c.1935C>A, c.1958C>A, c.2238G>C, and c.2815_2816del) GAA mutations. We have also identified two previously described

mutations, c.569G>A (p.R190H) [27] and c.1781G>A (p.R594H) in our patients [28].

To date, approximately 453 different sequence variants in the GAA gene are listed in the Pompe disease mutation database (www.pompecenter.nl). Numerous sequence variants have been demonstrated to be pathogenic. The most frequent mutation

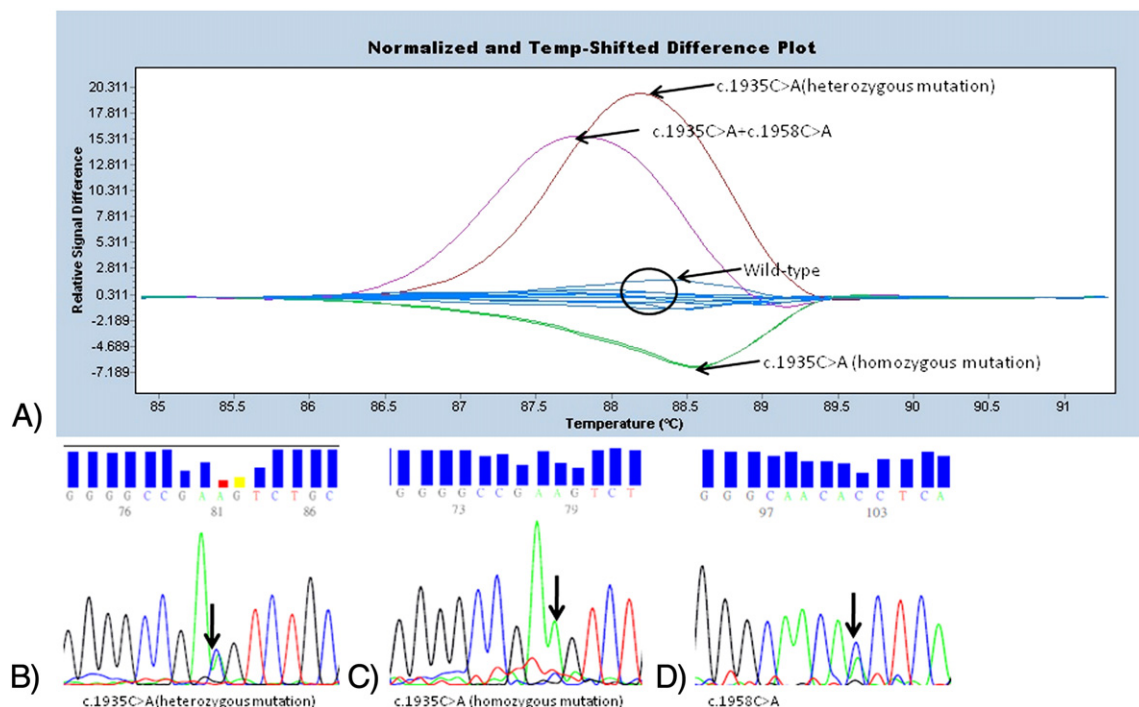


Fig. 4. Optimization of HRM curve analysis specific for c.1935C>A. (A) Normalized and temperature-shifted difference plots, the melting profile of c.1935C>A (homozygous mutation, heterozygous mutation, and wild-type) and c.1935C>A + c.1958C>A; electropherogram of mutation: (B) c.1935C>A (heterozygous mutation); (C) c.1935C>A (heterozygous mutation); (D) c.1958C>A (heterozygous mutation).

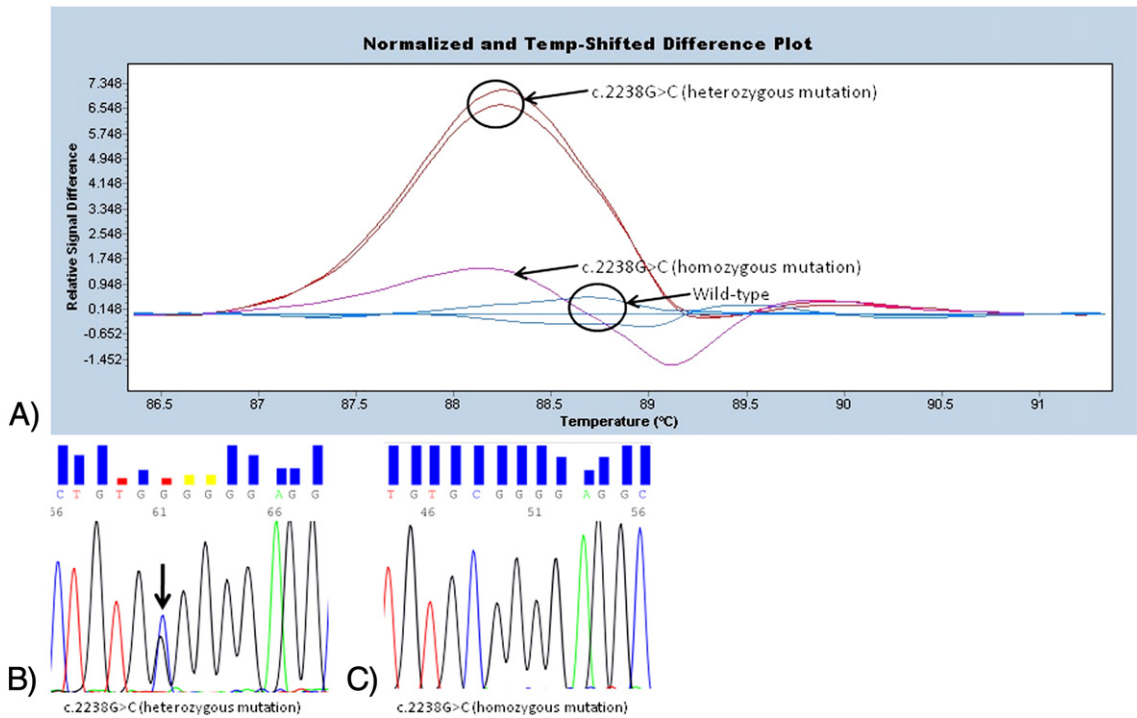


Fig. 5. Optimization of HRM curve analysis specific for c.2238G>C. (A) Normalized and temperature-shifted difference plots, the melting profile of c.2238G>C (homozygous mutation, heterozygous mutation, and wild-type); electropherogram of mutation: (B) c.2238G>C (heterozygous mutation); (C) c.2238G>C (heterozygous mutation).

among late-onset Pompe disease is the leaky c.-32-13T>G, which gives rise to alternatively spliced transcripts, including a deletion of the first coding exon, but still allows the production of a low amount of normally processed mRNA [1]. The c.-32-13T>G mutation was found over 40% patients of Caucasian origin [29–33]. In contrast, the c.-32-13T>G mutation has not been found among Asian population and this may indicate that the mutation shows ethnic specificity. Among the Taiwanese population, Yang et al. [34] revealed that two dual mutations in the GAA gene c.1935C>A; c.1726G>A and c.2238G>C; c.1726G>A represented 66.5% of the mutated chromosomes. Besides, Wan et al. [24] demonstrated that, the c.1935C>A (p.D645E) mutation was identified in 57.7% of both infantile and juvenile-onset GSD II. The author suggested that the c.1935C>A (p.D645E) mutation should be detected first when one suspects a GSD II patient. The mutation, c.1935C>A (p.D645E) has also been observed in up to 80% of infantile

cases. It is associated with a haplotype and suggested as a founder effect [35]. Meanwhile, Amarithnukrowh et al. [36] revealed that the mutation, c.1935C>A (p.D645E) accounts for 80% of the GAA mutations in Thai patients with infantile-onset Pompe disease. In the current study, both of the patients were compound heterozygous for this mutation. Notably, the reported mutation, c.1781G>A (p.R594H), is probably pathogenic because it involves the conserved residue during the evolution suggesting its essential functional role (Fig. 6). However, larger scale control experiment or further functional experiment may be necessary.

It should be noted that, the pseudodeficiency mutation, c.1726G>A (p.G576S), was found to be associated with severe mutation. The allele frequency of pseudodeficiency mutation among Taiwanese population is 14.5% [37]. It is far higher than that of European and sub-Saharan/African populations [38]. Also, the pseudodeficiency mutation may decrease the residual GAA activity of another mutation

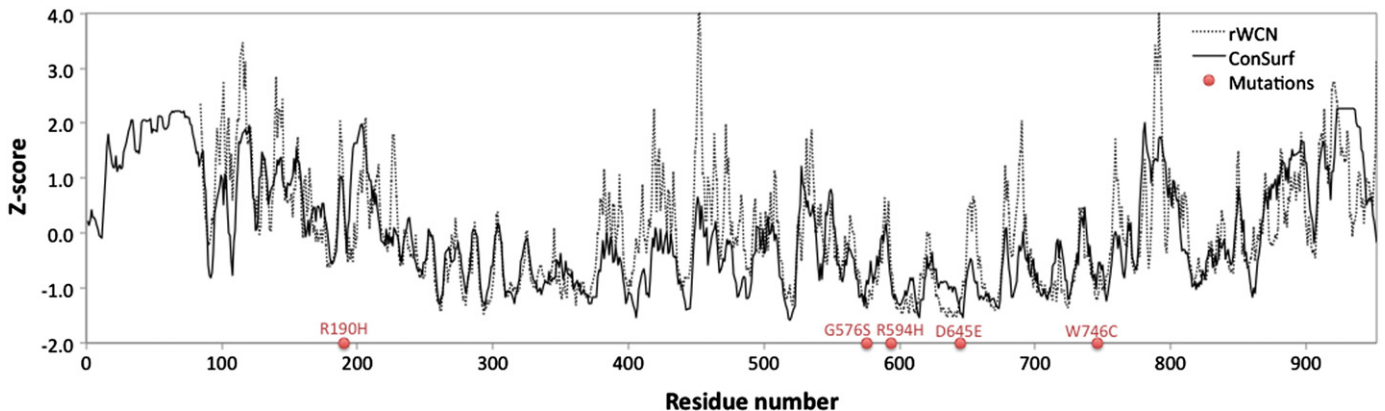


Fig. 6. The rWCN profile (dotted line) and ConSurf profile (solid line) of the GAA protein. The GAA mutations are marked in red circles. Both the rWCN and the conservation scores are normalized to their respective z-scores.

[39]. This may be the reason that Chinese patients with later-onset Pompe disease have earlier symptom onset than white patients. Indeed, both patients harbored the pseudodeficiency mutation having earlier symptom in the present study (Fig. S5). Aforementioned, two dual mutations in the *GAA* gene c.1935C>A; c.1726G>A and c.2238G>C; c.1726G>A were represented in 66.5% of the mutated chromosomes in the Taiwanese population [34]. On the other hand, the c.2238G>C (p.W746C) was shown to be associated with juvenile-onset GSD II and this mutation leads to a moderate disease phenotype. In this study, we designed three of primer-specific pairs for identifying c.1726G>A (p.G576S), c.1935C>A (p.D645E), and c.2238G>C (p.W746S) using HRM analysis (Supplementary data-1). The melting curve data showed the difference in normalized temperature-shifted data between the homozygous mutation, heterozygous mutation, and wild-type samples (Figs. 3–5). Once Pompe disease is suspected, physicians can screen these *GAA* mutations using HRM analysis.

Recently, Lobato et al. [32] developed the real-time PCR only for the detection of c.-32T>G (IVS1-13T>G) mutation of Pompe disease from DBS specimen. Besides, Pittis et al. [31] revealed 28 polymorphisms spread over the *GAA* gene using denaturing high performance liquid chromatography (DHPLC) analysis. The authors indicated that the c.-32-13T>G was the most frequent mutation, present as compound heterozygote in Italian population. Besides, the RT-PCR and long range-RT-PCR have also been used for the identification of *GAA* mutation in Pompe disease [34,40]. Notably, Chou et al. [41] demonstrated that HRM analysis had better sensitivity and specificity than DHPLC with the added advantages that some homozygous sequence alterations could be identified. Indeed, we could differentiate the heterozygous, homozygous, and wild-type DNA samples in the current study (Figs. 3–5). Till very recently, most screening approaches are now utilizing the more sensitive techniques of massively parallel sequencing. It has revolutionized genomics, providing a wide range of novel application on a high throughput, genome wide level. Although massively parallel sequencing has become the promising tool in genetic and genomic analysis, this approach is more expensive and the large amount of bioinformatics data creates informatics challenges. Meanwhile, it is not affordable for a small to the middle scale laboratory. In contrast, equipment, labor, reagent, and supply costs for analysis using the HRM analysis are substantially lower than the costs associated with analysis using massively parallel sequencing [42]. The HRM analysis is faster and less expensive than next-generation sequencing, facilitating rapid analysis of disease-causing mutations especially the high frequency of hotspot mutations.

In the current study, we also intended to model the proteins encoded by *GAA* to understand the structural implications of the mutations identified in this study. The atomic coordinates of the model structure were predicted using maltase-glucoamylase (PDB entry: 2QMJ) [23] as the template. The model of human *GAA* protein is shown in Fig. 7. The mutations of *GAA* are spread over the protein structure and distributed from the core to the surface of the enzyme molecule. A study showed that the substitution of G576 with S causes a small conformational change of side chains and does not affect the active site of the enzyme. The expressed protein may be unstable but exhibits considerable residual activity and normal affinity for substrates [39]. The structure analyses also reveal that both residues R190 and R594 have the salt bridge interactions with residues D253 and D860, respectively. A salt bridge is actually a combination of two non-covalent interactions: hydrogen bonding and electrostatic interactions. This is commonly observed to contribute stability to the proteins. Therefore, the substitution R190 with H or substitution D594 with H may reduce the salt bridge interaction and then destabilize the structure of *GAA*. The D645 is a conserved residue and is located at the catalytic domain. As previously described, the D645E substitution results in a decrease in enzyme activity and accounts for the observed defects in transport, phosphorylation and,

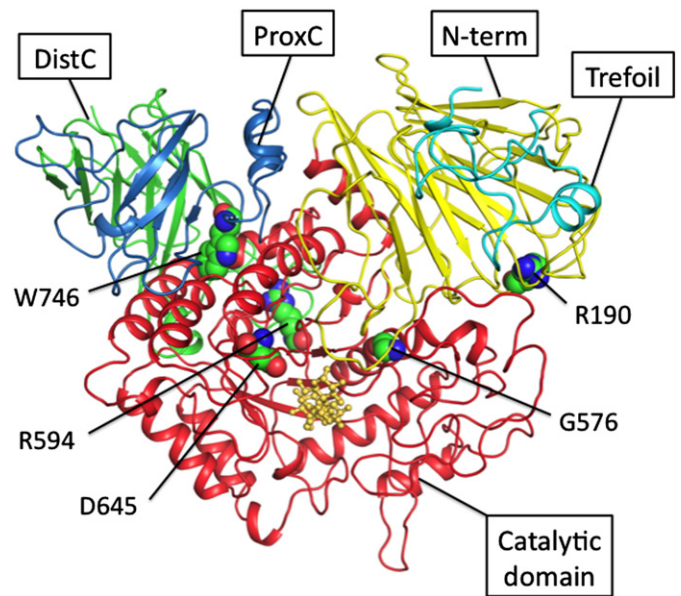


Fig. 7. Molecular modeling of Human *GAA*. The predicted wild type of human *GAA* model. The predicted structure comprises five domains: trefoil type P domain (light blue), N-terminal (yellow), catalytic domain (red), proximal C (blue), terminal and distal C terminal (green). The five residues R190, G576, R594, D645, and W746 are shown in a sphere mode. The 3D molecular graphs are displayed using PyMOL.

proteolytic processing of the newly synthesized α -glucosidase precursor of the patient [43]. The W746 residue is located at the proximal C terminal. Tryptophan (W) is unique in terms of chemistry and size. The substitution of W746 to S, a polar uncharged size change, may lead to a deleterious effect on protein function and structure.

In summary, we have demonstrated that HRM for mutation scanning of the *GAA* in patients with Pompe disease is a feasible and efficient method. This method differentiated all 7 known mutations (c.2T>C, c.1726G>A, c.1845G>A, c.1935C>A, c.1958C>A, c.2238G>C, and c.2815_2816del). Accordingly, HRM analysis is proved to be a workable, sensitive and in-tube methodology to scan *GAA* mutations in clinical practice. As aforementioned, the mutational analysis serves as an important role in establishing a definitive diagnosis especially in genotype–phenotype correlations in patients with Pompe disease. Therefore, we suggest that patients who are suspected of having Pompe disease should receive *GAA* gene analysis via HRM and direct sequencing for further diagnosis and appropriate treatments.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.cca.2013.10.013>.

Competing interests

The authors report no conflict of interest.

Authors' contributions

Concept and design of the experiments: TKE, CCC, YHC, WCL, and YJJ. Perform the experiments: TKE and CCC. Data analysis and discussion: TKE, CCC, WCL, YHC, and YJJ. Contribution of reagents/materials/analysis tools: CCC, TMK and YJJ. Clinical information: WCL, YHC and YJJ. Manuscript preparation: TKE, CCC, WCL, YHC, and YJJ. All authors read and approved the final manuscript.

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