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Clinical Laboratory Experience of Blood CRIM Testing in Infantile Pompe Disease

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Abstract

Cross-reactive immunological material (CRIM) status is an important prognostic factor in patients with infantile Pompe disease (IPD) being treated with enzyme replacement therapy. Western blot analysis of cultured skin fibroblast lysates has been the gold standard for determining CRIM status. Here, we evaluated CRIM status using peripheral blood mononuclear cell (PBMC) protein. For 6 of 33 patients (18%) CRIM status determination using PBMC was either indeterminate or discordant with *GAA* genotype or fibroblast CRIM analysis results. While the use of PBMCs for CRIM determination has the advantage of a faster turnaround time, further evaluation is needed to ensure the accuracy of CRIM results.

Keywords

acid alpha-glucosidase; cross reactive immunological material; Western blot analysis; Pompe disease; enzyme replacement therapy

Introduction

Pompe disease is caused by a deficiency of the lysosomal enzyme, acid alpha-glucosidase (GAA; EC 3.2.1.20) [1]. Patients with a severe deficiency of GAA activity present in infancy with cardiomyopathy and skeletal myopathy [1-3]. Cross-reactive immunological material (CRIM) status is an important prognostic factor for patients with infantile Pompe disease (IPD) being treated with enzyme replacement therapy (ERT) [4, 5]. About 30% of patients with IPD make no detectable GAA protein based on Western blot analysis using skin fibroblasts, and are classified as CRIM-negative [5, 6]. These patients usually fare poorly due to the development of high, sustained anti-rhGAA IgG antibodies that

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significantly reduce the efficacy of ERT. In contrast, CRIM-positive patients with IPD make some residual GAA protein, although with severely reduced or deficient GAA activity. They usually have low antibody titers and a better clinical outcome, however a subset of CRIM-positive cases also develop high sustained antibody titers [4]. Immune tolerance induction (ITI) for CRIM-negative patients, which prevents the production of anti-rhGAA IgG antibodies, is most effective when initiated in the ERT-naïve setting with the first dose of ERT [7]. This observation, coupled with the knowledge that early treatment in IPD results in the best prognosis, has necessitated development of a rapid method for determining CRIM status. Historically, CRIM status for IPD patients has been determined using Western blot analysis of skin fibroblast lysates; different bands representing inactive precursor (110 kDa), intermediate form (95kDa), and active forms (76 and 70 kDa) of GAA can be distinguished in normal skin fibroblast protein [8-11]. However, this process takes several weeks due to the time needed for culturing skin fibroblasts from skin biopsy tissue. In addition, some patients may make small amounts of GAA protein that are below the limits of detection by Western blot [6]. Recently, we have published that CRIM status can be predicted based on *GAA* gene mutations for over 90% of patients [12]. For example, premature stop codons caused by nonsense or frameshift mutations usually result in CRIM-negative alleles, and missense mutations typically result in CRIM-positive alleles. However, these predictions depend on the specific mutation, and prediction of CRIM status may not be possible or accurate for novel mutations in all patients. Therefore, a fast, accurate method of CRIM determination for all IPD patients would be beneficial. A new method for determining CRIM status, using peripheral blood mononuclear cells (PBMCs) has been reported recently [13], with promising results in a small group of patients. Here, we present results obtained using the same method to determine CRIM status in 33 IPD patients and comparison of those results with CRIM status predicted by *GAA* mutations, and/or CRIM status determined using skin fibroblasts, where available.

Patients and Methods

All subjects were diagnosed with Pompe disease and were enrolled in a Duke Medicine Institutional Review Board-approved protocol (IRB#Pro00001562; LDN6709 Site 206; Clinicaltrials.gov Identifier: NCT01665326). Samples for analysis were sent from across the USA to the Duke Biochemical Genetics Laboratory. CRIM status was determined by Western blot analysis of PBMCs and cultured skin fibroblast protein, as previously published [12, 13]. Specifically, blood samples for PBMC analysis were collected in BD Vacutainer® CPT™ (Cell Preparation Tube) with Sodium Citrate (Becton Dickinson, REF 362760, Franklin Lakes, New Jersey). Upon arrival in the laboratory, PBMCs were isolated within 24-48 hours of sample collection when possible. Cell lysates were prepared and Western blot analysis performed as previously described [13]. None of the patients in this study were on ERT at the time of sample collection. Protein samples from normal human fibroblasts and normal human PBMCs and known affected Pompe patients were used as the assay control for comparison (Corielle Institute for Medical Resarch, Camden, NJ). β-actin antibody was used as assess loading control and to assess the integrity of the patient protein samples [13].

Sequence analysis of the *GAA* gene (NM_000152) was performed in a CLIA and CAP certified laboratory. To predict CRIM status from *GAA* mutations, we used our database which correlates *GAA* mutations and CRIM status of about 140 patients with IPD [12]. If a mutation had been previously found in a CRIM-negative patient, it was designated as CRIM-negative. If a mutation had previously been found in homozygous state in a CRIM-positive patient, it was designated as CRIM-positive. If a mutation was not in our database, or was in a compound heterozygote CRIM-positive patient, we made the prediction of CRIM status as previously described [12]. Mutations resulting in a premature stop codon (nonsense, frameshift) are expected to be CRIM-negative, unless the stop codon occurs in the last exon of the gene or up to about 50 nucleotides from the 3'end of the penultimate exon, where it can be missed by the nonsense-mediated decay machinery [14]. Missense mutations are usually CRIM-positive unless the nucleotide affects a splice junction, or the change is in the first ATG codon of the gene, which can result in CRIM-negative status. The effect of splice site mutations can be difficult to predict unless the mutation has previously been associated with CRIM status, or in vitro studies of protein production have been done.

Results

Thirty-three patients were included in this study. Results of Western blot analysis of normal PBMC protein were comparable to those previously reported [13] (see Supplementary Material). In 27 of the 33 patients (82%), the CRIM status in PBMCs was concordant with the CRIM status predicted by *GAA* gene mutations (20 CRIM-positive, 7 CRIM-negative). For 8 of these 27 patients, skin fibroblast CRIM status was also available and was concordant with CRIM status in PBMCs and CRIM status predicted by *GAA* gene mutations (7 CRIM-positive, 1 CRIM-negative). For the remaining 6 patients (18%), CRIM status determination using PBMCs was either indeterminate or discordant with that determined by skin fibroblasts analysis and/or as predicted by *GAA* gene mutations (Table 1; see Supplementary Material). For five of these cases (Patients 1-5), a ~90kDa band was observed on Western blots of PBMC protein; four of these patients (Patients 1-4) were found to be CRIM-negative on skin fibroblast Western blot analysis and/or by prediction based on *GAA* gene mutations. Patient 5 was predicted to be CRIM-positive based on *GAA* gene mutations, and was confirmed to be CRIM positive by Western blot analysis of fibroblast protein. For the remaining one patient with discordant results (Patient 6), there were no bands in the size range of GAA protein on Western blot analysis of PBMC protein, but the patient was predicted to be CRIM-positive based on *GAA* genotype. Western blot using an anti β-actin antibody indicated that the protein was intact. A skin fibroblast sample from Patient 6 was not available for Western blot analysis.

Discussion

Development of a rapid method for determining CRIM status in patients with IPD is important so that appropriate treatment can be initiated as soon as possible. A blood-based method using PBMC for determination of CRIM-status would have the advantage of faster turnaround time as compared to use of cultured skin fibroblasts, the current gold standard [13]. However, our results indicate that the results of CRIM status determined by Western blot analysis of PBMC may not always be clear or concordant with those obtained by

analysis of skin fibroblast protein and CRIM status predicted from *GAA* gene mutations. The reason for this discrepancy in a subset of patients (18% in this cohort) is unclear. All PBMC samples in this study were prepared using the same standard protocol, and processed within 24-48 hours of collection. Western blot analysis with an anti β-actin antibody indicated that the protein was intact for all the discrepant samples.

Several PBMC samples were not included in this study because the protein sample was degraded and the results could not be considered reliable. This emphasizes the challenges of handling and shipment of these samples from distant places. The need for good internal controls to assess protein integrity and quality, and methods to stabilize protein during transportation cannot be emphasized enough. Additional challenges for the PBMC assay include the need for specialized cell preparation tubes (CPT), and limitations to obtaining sufficient blood sample volumes from patients who may be very young or fragile.

It is possible that the 90kDa band observed on Western blot analysis of PBMCs in Patients 1-5 may represent non-specific binding of the anti-GAA antibody. However, why this should occur in only some and not all samples is unknown. This band is particularly troubling because it is very close to the size range of GAA protein band (95 kDa). In Patient 6 a band of about 60 kDa, smaller than the expected size for GAA protein, was observed. There was no evidence of degradation of the sample on β-actin analysis. Of interest, this 60 kDa band has been observed in a previous study as well [13].

In conclusion, while a blood-based assay to determine CRIM status has the advantage of rapid results for faster initiation of treatment, further method development is needed to ensure the accuracy of the results. Important future work should include analysis of the 90 kDa and 60KDa bands that are present in some samples, but which do not correspond to any known GAA processing forms. Isolation and sequencing of this protein will be essential to determine whether its presence on Western blot is due to non-specific antibody binding, or whether it represents a breakdown product of GAA in PBMCs. Additionally, before initiating ERT, CRIM results obtained by analysis of blood PBMC should be confirmed by correlation with CRIM status as predicted by *GAA* mutations. Now that newborn screening for Pompe disease is a reality, more presymptomatic and early symptomatic cases are being identified. *GAA* mutation analysis is important not only for confirmation of the diagnosis in these cases, but also helpful in determining CRIM status in conjunction with Western blot results in PBMCs and/or fibroblasts if possible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CRIM	Cross-reactive immunological material
GAA	acid alpha-glucosidase
IPD	infantile Pompe disease
ITI	immune tolerance induction
PBMCs	Peripheral blood mononuclear cells

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Table 1

CRIM status in PCMCs and fibroblasts, and *GAA* mutations, in 6 patients with inconclusive PBMC CRIM status results.

Patient	CRIM status in PBMC	CRIM status in skin fibroblasts	Predicted CRIM status based on *GAA* mutations	*GAA* mutations	
				Allele 1	Allele 2
1	Indeterminate (90 kDa band)	Negative	Negative	c.437delT (p.Met146ArgfsX7)[a]	c.2237G>A (p.Trp746X)[b]
2	Indeterminate (90 kDa band)	Negative	Negative	c.1754+2T>A[c]	c.1822C>T (p.Arg608X)[d]
3	Indeterminate (90 kDa band)	Negative	Negative	c.2560C>T (p.Arg854X)[e]	c.2560C>T (p.Arg854X)[e]
4	Indeterminate (90 kDa band)	NA	Negative	c.2560C>T (p.Arg854X)[e]	c.2560C>T (p.Arg854X)[e]
5	Indeterminate (90 kDa band)	Positive (~110 kDa band)	Positive	c.1827delC (p.Tyr609X)[f]	c.2481+102_2646+31del (p.Gly828_Asn882del)[g]
6	Negative (60 kDa band)	NA	Positive	c.2297A>C (p.Tyr766Ser)[h]	c.2297A>C (p.Tyr766Ser)[h]

Further references and information about previously published mutations are available at www.pompecenter.nl/ (Pompe Center at Erasmus Medical Center).

[a] p.Met146ArgfsX7 is predicted to create a CRIM-negative allele due to introduction of a premature stop codon. To our knowledge, this mutation has not been found in other patients.

[b] c.2237G>A (p.Trp746X) was first reported by Beesley et al [15], and is predicted to create a CRIM-negative allele due to introduction of a premature stop codon. It was previously found in patients who were CRIM negative on fibroblast analysis [7 and 12].

[c] To our knowledge, c.1754+2T>A has not been found in any other patients. However, we have previously identified c.1754+1G>A in a patient who was CRIM-negative on fibroblast analysis, suggesting that abolishment of this splice site could result in a CRIM-negative allele [12]

[d] c.1822C>T (p.Arg608X) was previously reported as a “severity class A” mutation with no predicted expression of the protein [16].

[e] c.2560C>T (p.Arg854X) is common among patients with Pompe disease of African descent [17-20]. In cDNA studies, the allele carrying this mutation was found not to be expressed [21]. Patients who are homozygous for this mutation have been reported to be CRIM negative [7, 22, 23].

[f] 1827delC (p.Tyr609X) [24] is predicted to create a CRIM-negative allele due to introduction of a premature stop codon.

[g] p.Gly828_Asn882del is common among patients with Pompe disease of Dutch ancestry and is also found in other populations [25-30]. Previous studies show that this allele is transcribed and produces protein [12, 29].

[h] To our knowledge, p.Tyr766Ser has not been previously reported. We have found p.Tyr766Ser in homozygosity in three patients who are CRIM-positive, but not in any CRIM-negative patients (unpublished data).

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