Targeted gene panel screening is an effective tool to identify undiagnosed late onset Pompe disease

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Abstract

Mutations in the *GAA* gene may cause a late onset Pompe disease presenting with proximal weakness without the characteristic muscle pathology, and therefore a test for *GAA* activity is the first tier analysis in all undiagnosed patients with hyperCKemia and/or limb-girdle muscular weakness. By using MotorPlex, a targeted gene panel for next generation sequencing, we analyzed *GAA* and other muscle disease-genes in a large cohort of undiagnosed patients with suspected inherited skeletal muscle disorders (n = 504). In this cohort, 275 patients presented with limb-girdle phenotype and/or an isolated hyperCKemia. Mutational analysis identified *GAA* mutations in ten patients. Further seven affected relatives were identified by segregation studies. All the patients carried the common *GAA* mutation c.-32-13T >G and a second, previously reported mutation. In the subcohort of 275 patients with proximal muscle weakness and/or hyperCKemia, we identified late-onset Pompe disease in 10 patients. The clinical overlap between Pompe disease and LGMDs or other skeletal muscle disorders suggests that *GAA* and the genes causing a metabolic myopathy should be analyzed in all the gene panels used for testing neuromuscular patients. However, enzymatic tests are essential for the interpretation and validation of genetic results.

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1. Introduction

Glycogen storage disease II or Pompe disease (MIM 232300) is an autosomal recessive disorder caused by mutations in the *GAA* gene (MIM 606800), encoding alpha-1,4-glucosidase, a lysosomal enzyme involved in the degradation of glycogen [1,2].

Depending on the level of residual enzymatic activity, mutations in *GAA* lead to a wide spectrum of clinical phenotypes, ranging from a severe infantile form, presenting with cardiomyopathy and muscular hypotonia, to a relatively milder, late onset form of Pompe disease (LOPD), characterized by a slowly progressive proximal muscle weakness, often involving the respiratory muscles [3–5].

The diagnosis of LOPD is challenging and is usually achieved with significant delay. LOPD patients, in fact, may exhibit a mild phenotype, partially overlapping that of other neuromuscular disorders (NMDs) [2,6]. Moreover, the histopathological findings are often unspecific, given the fact that often neither glycogen storage nor vacuoles are present in LOPD muscle biopsies [7,8]. The clinical overlap with other disorders and the misleading histological features often hamper an early diagnosis at onset of the symptoms, resulting in a diagnostic odyssey [6,8].

The measurement of *GAA* activity, usually on a dried blood spot (DBS), serves as diagnostic screening test [9], al-though the results must be confirmed by a second biochem-ical assay on different tissues as skeletal muscle, fibroblasts or leucocytes and corroborated by the presence of two *GAA* pathogenic mutations [10,11].

A disease-specific treatment, the enzyme replacement therapy (ERT), is available since 2006. However, ERT has a limited effect on LOPD patients [12]. Early diagnosis and the subsequent early initiation of therapy may have an impact on the therapeutic success, preventing severe muscular and respiratory impairment [6].

In recent years, the development of cutting-edge technologies has revolutionized the diagnostic approach to the genetic disorders [13]. Different NGS approaches for the diagnosis of patients with skeletal muscle diseases have been described in literature [14]. In particular, targeted resequencing (TGS) of specific genes of interest represents a cost-effective strategy for the clinical diagnostics of heterogeneous disorders [14,15].

In this manuscript, we describe clinical and morphologi-cal data of 16 LOPD patients identified by performing MotorPlex, an NGS-based platform to analyze genes associated with skeletal muscle disorders, with subsequent segregation analysis in a large cohort of myopathy patients.

2. Materials and methods

DNA samples from 504 patients with a broad range of myopathies, including LGMDs, congenital myopathies, distal myopathies, and isolated hyperCKemia, were recruited in the network of the Italian Association of Myology. Patients were clinically evaluated by neurologists within tertiary care centers for neuromuscular disorders and had remained without final diagnosis. For each patient, clinical history and laboratory data were collected in a specific form. Patients signed a written informed consent at the time of blood collection. The study was approved by the Ethics Committee of Univer-sità degli Studi della Campania "Luigi Vanvitelli" and was performed according to the Declaration of Helsinki.

Genomic DNA was extracted from peripheral blood by phenol/chloroform extraction. All the samples were enriched using MotorPlex [16,17], a custom assay to analyze a panel of genes causing neuromuscular disorders. All the enrichments were performed according to manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA). Each library was run on a HiSeq 1000 (Illumina).

An in-house pipeline [18] was used to analyze the raw sequencing data generated. All the variants were confirmed by Sanger sequencing using an ABI PRISM 3130 XL automatic DNA Sequencer Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

A segregation analysis was performed according to the availability of DNA from relatives.

A biochemical test evaluating the *GAA* activity was performed, when possible, on samples from patients with *GAA* mutations. *GAA* activity in skeletal muscle, DBS and lymphocytes was assessed by a fluorometric method using the substrate 4-methylumbelliferyl-α-D-glucoside [19]. The newly diagnosed LOPD patients were re-assessed, re-evaluating the clinical presentation, functional measures (6 minute walking test, 6MWT), creatine kinase levels and respiratory functions (forced vital capacity, FVC, in supine and/or seated position).

We performed a copy number variant (CNV) analysis by using a custom array-CGH, MotorChip (Agilent Technologies) [20].

3. Results

Sixteen patients from nine unrelated families were diagnosed with LOPD (patient II has been extensively described elsewhere [21]). All patients showed the common c.-32-13T >G variant [22] and a second, already reported, mutation on the other allele [23–27] (Table 1). In particular, the sec-

Table 1
Patients with late-onset pompe disease.

Case	Onset	Age at diagnosis (y)	Symptoms at onset			Last assessment					Genotype (NG_009822.1 or NM_000152.3)	
			Limb weak.	Resp.	Other	6MWT (mt)	Plasma CK	FVC seated ml (%N)	Histology	*GAA* activity (pmol/min/mg)	1st allele	2nd allele
I	early adul.	49	prox.	no	fatigability	n.a.	5–10×	n.a.	myopathic	lymphocytes: 14.2	c.784G >A;p.E262K [23]	c.-32-13T >G [18]
II*	adul.	71	prox.	no	myalgia	40	5–10×	850 (22%)	myopathic	DBS: 0	c.1564C >G;p.P522A [27]	
III	adul.	63	prox. and dist.	no	fatigability, dysphagia	n.a.	2.5–5×	n.a.	myopathic	muscle: 11.7	c.1927G >A;p.G643R [25]	
IV,1	early adul.	40	prox.	yes	exercise intolerance	n.a.	2.5–5×	n.a.	vacuoles	n.a.	c.1124G >T;p.R375L [24]	
IV,2	early adul.	47	prox.	yes		n.a.	2.5–5×	n.a.	n.a.	n.a.		
IV,3	early adul.	50	not observed	no		n.a.	2.5–5×	n.a.	n.a.	n.a.		
IV,4	early adul.	45	prox.	no		n.a.	2.5–5×	n.a.	vacuoles	n.a.		
V,1	adol.	42	prox.	no	fatigability, myalgia	135	5–10×	950 (20%)	vacuoles	DBS: 0.20	c.1124G >T;p.R375L [24]	
V,2	adul.	33	prox.	no	fatigability	566	5–10×	3000 (90%)	n.a.	DBS: 0.20		
V,3	child.	45	prox.	no	n.a.		2.5–5×	n.a.	n.a.	DBS: 0.08		
VI,1	early adul.	63	prox. and axial	yes	fatigability, myalgia	n.a.	normal	1520 (38%)	n.a.	DBS: 0.24	c.2237G >A;p.W746* [26]	
VI,2	adul.	61	prox.	yes	fatigability	418	2.5–5×	2040 (46%)	myopathic	DBS: 0.22		
VI,3	adul.	64	prox.	yes		n.a.	2.5–5×	1550 (48%)	n.a.	DBS: 0.16		
VII	adol.	33	prox. and dist.	no	cramps	n.a.	2.5–5×	5260 (112%)	vacuoles	DBS: 0.20	c.784G >A;p.E262K [23]	
VIII	early adul.	56	prox.	no	fatigability, myalgia	385	2.5–5×	2480 (75%)	myopathic	DBS: 0.13	c.G989A;p.W330* [27]	
IX	early adul.	n.a.	prox.	yes	fatigability, cardiac involvement	WCB	n.a.	n.a.	n.a.	n.a.	c.1124G >T;p.R375L [24]	

y = years; Resp. = respiratory; 6MWT = 6 minutes walking test; N = normal; adult. = adulthood; child. = childhood; adol. = adolescence; prox. = proximal; dist. = distal; n.a. = not available; DBS = dried blood spot; CK = creatine kinase; FVC = forced vital capacity; WCB = Wheel-chair-bound.

* Patient II has been described in full elsewhere [21].

ond allele carried a missense change in seven families and a nonsense variant in the remaining two families.

Among LOPD patients, there were more men (10/16, 62.5%) than women (6/16, 37.5%), with a male to female sex ratio of 1.66. Adult onset was observed in almost all patients (13/16, 81.2%), except patients V,1 and VII who showed their first symptoms during adolescence and patient V,3 who had a childhood onset.

The symptoms at onset were mainly proximal weakness (12/16, 75%), and fatigability (10/16, 62.5%). Exercise intolerance, myalgia and contractures were less common in this cohort; some of the patients showed dysphagia. None of the patients had central nervous system involvement at onset; the heart was affected in only one patient with an early adult presentation. Respiratory involvement was observed during the first visit in six patients (37.5%). Increased creatine kinase levels (2.5–10× the upper normal limit) were observed in 14 patients. When performed (6 cases), electromyography showed a myopathic pattern. Muscle biopsy, performed in 9 patients, showed presence of vacuoles in only four patients.

The mean age of the patients was 50.8 ± 11.7 years (range 33–71). The average diagnostic delay was 21.5 years (range = 11–38). Two patients used a wheelchair at the time of latest evaluation. *GAA* enzymatic activity, tested in 11 patients (9 through DBS, 1 each in muscle and lymphocytes) was reduced in all samples, confirming the diagnosis of LOPD.

A marked inter- and intrafamiliar phenotypic variabilities were observed. The presence of a nonsense variant (families VI and IIX) *in trans* with the common c.-32-13T >G variant did not correlate with either a more severe phenotype or an earlier onset of the disease.

Our screening also identified five patients (three affected by LGMD and two by a congenital myopathy), carrying the c.-32-13T >G variant in heterozygosity. In two of them the identification of already described mutations addressed the diagnosis toward a different disease (LGMD2C and nema-line myopathy 1). In the three remaining patients, no other causative variants in *GAA* were identified and a custom array-CGH [20] excluded the presence of any copy number variants affecting this gene in two of the patients (the third patient was not tested because of DNA unavailability). Unfortunately the loss to follow-up of these patients did not allow us to perform any biochemical test to evaluate the *GAA* activity.

4. Discussion

We analyzed *GAA* together with all other genes included in the MotorPlex [15,16] in 504 genetically undiagnosed pa-

Table 2
Broadening the spectrum of clinical phenotypes associated with metabolic myopathies by next generation sequencing.

Reference	Genetic approach	Cohort	Results	Atypical elements
Ghaoui et al. [32]	WES	LGMD (n = 60)	1 *GAA*[*]	n.a.
			1 *CPT2*	fixed weakness
			1 *PYGM*	fixed weakness
Todd et al. [33]	WES	LGMD (n = 38)	1 *GBE*	fetal akinesia and multiple pterygium syndrome
Lévesque et al. [34]	Targeted resequencing	Muscle Disorders (n = 34)	1 *GAA*	long lasting history of gait disturbances
Kuhn et al. [35]	Targeted resequencing	LGMD (n = 58)	1 *GBE*	minimal non-lysosomal glycogen storage without any evidence of polyglucosan bodies
Reddy et al. [36]	WES	LGMD (n = 55)	1 *GAA*	n.a.
			1 *VCP*	
Johnson et al. [37]	WES	LGMD and/or hyperCKemia (n = 606)	8 *GAA*	n.a.

n.a. = not available; WES = whole exome sequencing.

[*] LOPD patient was identified in a pre-NGS screening.

tients who presented a wide spectrum of clinical phenotypes, ranging from isolated hyperCKemia to mild or severe muscular impairment, and a variable age of onset and disease progression [16]. Two-hundred-seventy-five patients (in 259 families), presenting with a limb-girdle muscular dystrophy (LGMD) and/or an isolated hyperCKemia, constituted a risk group for LOPD.

Within this subgroup, we identified ten patients in nine families carrying *GAA* mutations consistent with a diagnosis of LOPD. Seven affected relatives were subsequently identified by segregation analyses in these families. This corresponds to a prevalence of LOPD of 3.63% (10/275) in the undiagnosed patients with adult onset proximal weakness or hyperCKemia. As expected, no bi-allelic causative *GAA* variants were identified among patients with other phenotypes such as, congenital myopathies, congenital dystrophies, dis-tal myopathies, etc. Screening projects for Pompe disease by using DBS, in cohorts with similar phenotypes have been performed previously, achieving a similar results [9,28–30], the only exception is Finland where Pompe disease is extremely rare [31] due to lack of endemic founder mutations. Several factors have hampered an early diagnosis in all the reported cases. First, muscle biopsy showed not specific myopathic aspects in most cases. Second, the loss to follow-up of the patients for many years excluded an early detection of respiratory symptoms critical to raise the clinical suspicion of LOPD. Third, in most patients, the clinical presentation including atypical signs, e.g. dysphagia in patient III, pseudohypertrophy in patient VII or calf hypertrophy in patient VIII, was probably misleading. In patient I, a previous western blot analysis had showed a reduction of calpain 3 (*CAPN3),* and Sanger sequencing of *CAPN3* only revealed a heterozygous causative missense variant. At that time, the molecular findings may suggest the presence of a second elusive mutation, corroborating the preliminary diagnosis of LGMD2A. However, this patient was included in the study, because this second variant was truly absent from mRNA sequencing. This confirms that any genetic report should be very cautious in similar cases. Similarly, reduced staining for dystrophin in patient VIII suggested Becker dystrophy.

On the other hand, currently NGS-based strategies are largely adopted to study genetically heterogeneous conditions, including muscle diseases [14]. Recent NGS data prove that 20%–30% of myopathic patients carry causative mutations in genes typically not associated with the observed pheno-type [16,32–34]. Many studies [32–37] identified mutations in genes responsible for LOPD and other metabolic myopathies in patients with a wide range of phenotypes (Table 2). At the same time, a well-known homozygous mutation in *ANO5*, responsible for a wide spectrum of disorders described under the umbrella term “anoctaminopathy” [38,39], was identified in a patient suspected of having glycogen storage disease be-cause of the presence of glycogen containing vacuoles on muscle biopsy [40].

The decreasing cost and the technological improvements will make NGS approaches an effective first tier screening method in the near future [41,42]. Given the large heterogeneity of neuromuscular disorders and the increasing number of associated genes, Mendeliome or whole exome sequenc-ing are advisable. However, the genetic analysis has to be included within a standardized diagnostic algorithm comprising exhaustive clinical, histological and imaging tests which are essential for a proper interpretation of molecular findings. Consequently, biochemical assays, including DBS, and protein studies will remain essential for the interpretation and validation of genetic results [34]. Above all, tests for acid alpha-glucosidase activity are still the gold standard tests when clinicians suspect a Pompe disease in a patient. A smart, combined use of these tests and NGS will allow a rapid diagnosis and, thereby, an early initiation of available treatments.

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