

Molecular genetics of late onset glycogen storage disease II in Italy

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Glycogen Storage Disease Type II (GSDII) is a recessively inherited disorder due to the deficiency of acid α -glucosidase (GAA) that results in glycogen accumulation in the lysosomes. The molecular analysis of the GAA gene was performed on 45 Italian patients with late onset GSDII. DHPLC analysis revealed 28 polymorphisms spread all over the GAA gene. Direct sequencing identified the 96% of the mutant alleles, 12 of which are novel. Missense mutations were functionally characterized by enzyme activity and protein processing in a human GAA deficient cell line while splicing mutations were studied by RT-PCR and *in silico* analysis. A complex allele was also identified carrying three different alterations *in cis*. All the patients studied carried a severe mutation in combination with a milder one, which explains the late onset of the disease. The c.-32-13T > G was the most frequent mutation, present as compound heterozygote in 85% of the patients as described in other late onset GSDII Caucasian populations. Interestingly, 10 of the 45 patients carried the c.-32-13T > G associated to the severe c.2237G > A (p.W746X) mutation. However, despite the common genotype, patients presented with a wide variability in residual enzyme activity, age of appearance of clinical signs and rate of disease progression, suggesting that other genetic/environment factors may modulate clinical presentation.

Introduction

Glycogen Storage Disease Type II (GSDII; Pompe disease, acid maltase deficiency, MIM# 232300) is an autosomal recessive inherited disorder due to the deficiency of acid α -glucosidase (GAA; E.C.3.2.1.20) that results in impaired glycogen degradation which accumulates within the lysosomes.

Clinically, GSDII encompasses a continuous spectrum of phenotypes, from a rapidly progressive infantile form leading to death within the first year of life to a slowly progressive late-onset form of the disease that affects mobility and respiratory function. Classic infantile GSDII manifests soon after birth and is characterized by absent or nearly absent enzyme activity, severe muscle weakness, cardiomegaly/cardiomyopathy and respiratory insufficiency, that typically lead to death within the first year of life (1-4). Some infantile patients have less severe cardiac involvement without left cardiac output obstruction,

survive longer and die because of pulmonary infections with secondary ventilatory insufficiency (5, 6). Late onset GSDII comprises all milder subtypes: partial enzyme deficiency manifests in children and adults as slowly progressive skeletal muscle weakness without cardiac involvement. Respiratory muscle weakness, particularly of the diaphragm, is the leading cause of death in the late-onset cases. (1, 2, 4, 7-9).

The GAA gene (MIM# 606800) located in the human chromosome 17q25.2-25.3 produces an inactive 110 kD precursor which is transported to the lysosomal compartment and processed into the 95 kD intermediate and the fully active forms of 76 and 70 kD (1, 10-13). More than 200 mutations in the GAA gene have been described up to date (<http://www2.eur.nl/fgg/ch1/pompe>).

In an extensive collaborative study we analysed the complete mutational profile in 45 Italian patients affected by the late onset GSDII. We were able to characterize 27 mutant alleles leading to the identification of 12 novel mutations. Missense mutations were functionally characterized *in vitro* by enzyme activity and protein processing and splicing mutations were studied by RT-PCR or *in silico* analysis. This work offers a complete picture of the late onset GSDII molecular genetics in Italy which contributes in the understanding of the natural history and in the evaluation of emerging ERT efficacy.

Material and Methods

Patients

We studied 45 Italian patients with late onset GSDII (19 females and 26 males). The diagnosis was based on clinical data and confirmed by reduced GAA activity in lymphocytes or muscle. The age at diagnosis varied from 2 to 68 years. Almost all the patients underwent a program of physiotherapy, high protein diet and respiratory management in their reference centres. Most of the patients have had mild muscular symptoms since childhood. First complaints were mostly related to mobility problems, weakness and fatigue.

GAA mutation analysis

Genomic DNA was extracted with the QIAamp DNA blood Mini Kit (Qiagen GmbH, Hilden, Germany). *GAA* gene was amplified as described (14). PCR products were screened by denaturing High Performance Liquid Chromatography (dHPLC, Varian, Palo Alto, CA, USA) and in the presence of heteroduplex, sequenced on the ABI PRISM 3700 DNA Analyzer. Mutations were confirmed by sequencing duplicate PCR products and confirmed by the analysis of parental DNA.

For RT-PCR analysis the first strand cDNA was synthesized using random hexamer primers and subsequent amplification was done in six overlapping fragments as described by Hermans et al. (15).

All mutations are described according to mutation nomenclature, considering nucleotide + 1 the A of the first ATG translation initiation codon (16, 17, <http://www.hgvs.org/mutnomen>). Nucleotide numbers are derived from cDNA *GAA* sequence (RefSeq cDNA Y00839.1).

Site directed mutagenesis

Missense mutations were introduced in the wild type full length cDNA *GAA* cloned in pcDNA3 (Invitrogen) by site directed mutagenesis using the Quickchange Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX, USA). Each clone was entirely sequenced to confirm that no other mutations were introduced by the PCR-based mutagenesis procedure.

Table 1. Mutation profile of the *GAA* gene in the Italian late onset GSDII population.

Location	cDNA mutation*	Mutation effect	Allele frequency
intron 1	c.-32-13T > G	leaky splice	42.0%
exon 2	c.258dupC	p.N87QfsX9	1.1%
exon 2	c.525delT	p.E176RfsX45	3.4%
intron 3	c.692 + 1G > C	r.0	2.2%
exon 5	c.925G > A	p.G309R	1.1%
exon 6	c.1064 T > C	p.L355P	3.4%
intron 6	c.1076-1G > C	r.1076-79_1195 + 89ins	1.1%
exon 7	c.1082C > T	p.P361L	1.1%
intron 7	c.1194 + 2T > A	r.spl?	1.1%
exon 9	c.1331C > G	p.P444R	1.1%
exon 9	c.1333G > C	p.A445P	1.1%
exon 10	c.1465G > A	p.D489N	3.4%
intron 10	c.1551 + 1G > C	p.V480_I517del	1.1%
exon 11	c.1626C > G	r.spl?	2.2%
exon 12	c.1645G > C	p.G549R	1.1%
exon 12	c.1655T > C	p.L552P	2.2%
exon 13	c.1776del G	p.T593HfsX5	1.1%
exon 13	[c.1833_1839del;c.1846G > T; 1847_1848insT]	p.H612_D616delinsRGI	1.1%
exon 13	c.1836C > G	p.H612Q	1.1%
exon 14	c.1927G > A	p.G643R	1.1%
exon 14	c.2014C > T	p.R672W	3.4%
exon 15	c.2104C > T	p.R702C	1.1%
exon 16	c.2219_2220delTG	p.V740GfsX55	1.1%
exon 16	c.2237G > A	p.W746X	10.3%
exon 16	c.2242dupG	p.E748GfsX48	1.1%
intron 17/18	c.2481 + 102-2646 + 31del	p.G828_N882del	2.2%
exon 18/intron 18	c.2646_2646 + 1delTG	r.spl?	1.1%

* New mutations are indicated in **bold**; cDNA reference sequence Y00839.1. For cDNA numbering + 1 corresponds to the A of the first ATG translation initiation codon.

Cell culture and *in vitro* expression assay

Patient fibroblasts obtained from skin biopsies and the Ad5-SV40 immortalized human GAA-deficient fibroblast cell line were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine and 50 mg/ml penicillin/streptomycin (Gibco, Paisley, UK).

Cells were transfected with wild type and mutant constructs with a standard calcium/phosphate using 4 µg of total plasmid DNA Endofree purified (Sigma, St. Louis, MO, USA), harvested after 48 h and assayed for GAA activity and Western blot as described elsewhere (18).

Results and discussion

The mutation profile of the GAA gene was analysed in 45 patients with the late onset form of the disease. We

identified 27 different alleles corresponding to the 96% of the total alleles: 12 of them are novel including a complex allele that carried three different alterations *in cis* (Table 1). The GAA profile was characterized by all kind of mutations, including single base changes, both small and large deletions, small insertions and splicing aberrations (19).

Samples were first screened by DHPLC and subsequently sequenced, revealing 28 polymorphisms spread all over the GAA gene (Table 2). DHPLC technique allows an accurate and rapid mutation screening which reduces costs and working time but is not useful in the presence of highly polymorphic genes as the GAA.

The deleterious effect of the novel missense mutations was confirmed by *in vitro* expression analysis in human GAA-deficient fibroblasts transiently transfected with the wild type and mutant GAA. As shown

Table 2. GAA polymorphisms in Italian population.

Location	Single base change [§]	Protein Sequence	Frequency*
Exon 2	c.271GA	p.D91N	0.6%
	c.324TC	p.C108C	28%
Intron 2	c.547-4CG	Non coding	31%
	c.547-39TG	Non coding	25%
Exon 3	c.596GA	p.H199R	42%
	c.642CT	p.S214S	18%
	c.668AG	p.H223R	44%
Intron 4	c.854ins7nt + 7	Non coding	32%
	c.858 + 29TC	Non coding	15%
Exon 5	c.921AT	p.A307A	5.4%
Intron 5	c.955 + 12 AG	Non coding	33%
Exon 8	c.1203AG	p.Q401Q	3.9%
Intron 8	c.1327-18GA	Non coding	35%
Exon 9	c.1374CT	p.Y458Y	5.1%
Intron 9	c.1438-19CG	Non coding	42%
Exon 11	c.1581GA	p.R527R	32%
Intron 12	c.1754 + 12GA	Non coding	24%
Exon 13	c.1830CT	p.A610A	11%
Intron 14	c.2040 + 20AG	Non coding	36%
Exon 15	c.2133AG	p.T711T	15%
	c.2154TC	p.V718V	35%
Intron 15	c.2190-13GT	Non coding	40%
Exon 16	c.2238GC	p.W746C	10%
Intron 16	c.2331 + 20GA	Non coding	38%
Exon 17	c.2338AG	p.I780V	42%
Exon 18	c.2553GA	p.G851G	40%
Exon 20	c.3082CT	Non coding	24%
	c.3277TC	Non coding	30%

For cDNA numbering + 1 corresponds to the A of the first ATG translation initiation codon (cDNA reference sequence Y00839.1). [§] New polymorphisms are indicated in **bold**. * Frequency is calculated on 100 normal alleles

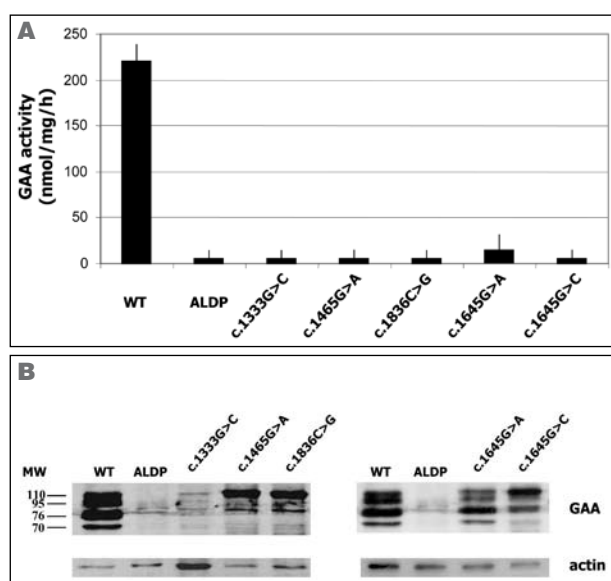


Figure 1. A) Enzyme activity in GAA deficient fibroblasts transiently transfected with the wild type pCDNA3-GAA and mutant constructs, measured using the fluorogenic substrate 4-MU-a-D-glucopyranoside. B) Western blot analysis of the cellular extracts described above. All GAA forms are detected in the wild type transfected cells (WT) while no GAA is detected in the mock transfected cells (ALDP). Almost all the protein obtained from the c.1465G > A and c.1836C > G constructs remained as the GAA inactive precursor of 110kD. The c.1645G > A mutant was correctly processed while c.1645G > C accumulates predominantly as the 110kD and 76kD forms. For the c.1333G > C construct, a faint band of 110 kD was detected when a higher amount of protein was loaded onto the gel, suggesting that the protein is highly unstable and it is rapidly degraded.

in Figure 1A, neither of the mutant proteins expressed residual enzyme activity. Moreover, Western blot analysis demonstrated that the expression pattern of the mutant proteins differed in all cases from the wild type GAA, which confirms they are disease causing mutations (Fig. 1B).

Only four patients had one of the two still unknown alleles. However, in one of these patients, the paternally inherited mutation (c.-32-13T > G) was observed as compound heterozygosity in genomic DNA and in apparent homozygosity in cDNA. Based on these findings we assumed that the unknown allele may harbor an unidentified mutation in the non coding regions of the GAA gene that prevents the formation of a stable mRNA.

The mutation profile of the GAA gene in Italian late onset GSDII patients was quite heterogeneous, similar to what has been previously described in the French late onset GSDII population (19). As described in the Caucasian late onset GSDII population the c.-32-13T > G resulted the most frequent mutation (allele frequency 42%) (2). In

all cases studied, the combination of known severe mutations with milder mutations explained the late onset of the disease. Interestingly, the c.-32-13T > G was associated to the severe c.2237G > A (p.W746X) in 10 of the 45 patients studied. Despite the common genotype, patients presented with a wide variability in residual enzyme activity, age of appearance of clinical signs and rate of disease progression.

This work represents the largest study of GSDII conducted in Italy to date. It should be pointed out that almost half of the mutant alleles found are due to novel mutations. Therefore, *in vitro* analysis resulted an useful tool in discriminating disease-causing mutations and evaluating their effect on the normal enzyme function.

Increasing knowledge on the mutant protein structure may be potentially used in the development of novel therapeutic strategies (Parenti, et al., in press). However, *in vivo* enzyme function determination is still preferable for genotype/phenotype correlation (20, 21).

Our data confirmed the wide spectrum of clinical manifestations observed in GSDII and the phenotypic variability among patients, even carrying the same genotype. Moreover, continued mutational analysis contributes in the understanding of genotype/phenotype correlations and this may be useful in the evaluation of emerging ERT efficacy.

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