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The GST domain of GDAP1 is a frequent target of mutations in the dominant form of axonal Charcot Marie Tooth type 2K

¹Claudia Crimella, ¹Alessandra Tonelli, ¹Giovanni Airoidi, ¹Cinzia Baschiroto, ²Grazia D'Angelo, ²Sara Bonato, ³Luciana Losito, ³Antonio Trabacca, ^{1,4}Nereo Bresolin, ¹Maria Teresa Bassi.

E. Medea Scientific Institute, ¹Laboratory of Molecular Biology, Bosisio Parini, Italy; ²Neuromuscular and Neurorehabilitation Unit, Bosisio Parini, Italy; ³E. Medea Scientific Institute, Unit of Neurorehabilitation I, Developmental Neurology and Functional Rehabilitation, Ostuni, Italy; ⁴Dino Ferrari Centre, IRCCS Ca' Granda, Ospedale Maggiore Policlinico Foundation, Dept. of Neurological Sciences University of Milan, Italy.

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Corresponding Author: Maria Teresa Bassi, PhD, Laboratory of Molecular Biology, E. Medea Scientific Institute, Via D. L. Monza 20, 23842 Bosisio Parini, Lecco, Italy; Phone 0039 0318771111, Fax 0039 031877499, email: mariateresa.bassi@bp.lnf.it.

Abstract

Background: Mutations in *GDAP1* associate with both demyelinating (CMT4A) and axonal (CMT2K) forms of CMT. While CMT4A shows recessive inheritance, CMT2K can present with either recessive (AR-CMT2K) or dominant segregation pattern (AD-CMT2K), the latter being characterized by milder phenotypes and later onset. The majority of the *GDAP1* mutations are associated with CMT4A and AR-CMT2K, with only 4 heterozygous mutations identified in AD-CMT2K.

Methods: We screened *GDAP1* gene in a series of 43 index patients, 39 with CMT2 and 4 with intermediate CMT, with both sporadic and familial occurrence of the disease. Results: Three novel mutations were identified in three families with dominant segregation of the disease: two missense changes, p.Arg226Ser and p.Ser34Cys, affecting the GST domain of the GDAP1 protein and a novel deletion (c.23delAG) leading to early truncation of the protein upstream the GST domain. Wide variability in clinical presentation is shared by all three families mostly in terms of age at onset and disease severity. A rare variant p.Gly269Arg, located within the GST domain, apparently acts as phenotype modulator in the family carrying the deletion. Conclusion: The results obtained reveal a *GDAP1* mutation frequency of 27% in the dominant families analyzed, a figure still unreported for this gene, thus suggesting that *GDAP1* involvement in dominant CMT2 might be higher than expected.

Introduction

Charcot-Marie Tooth disease (CMT) is an heterogeneous group of diseases characterized by chronic motor and sensory polyneuropathy leading to progressive loss of sensation, weakness, muscle atrophy, loss of deep tendon reflexes and foot deformities. CMT can be classified in two major subtypes: demyelinating CMT (CMT1, CMT4), associated with reduction in nerve conduction velocities (NCV) in all nerves and segmental demyelination and remyelination; and axonal CMT (CMT2) associated with normal or almost normal NCVs, loss of myelinated axons and regenerative sprouting. A CMT subgroup showing NCV values “intermediate” between CMT1 and CMT2 also exists. Mutations in the ganglioside-induced differentiation-associated protein 1 (*GDAP1*) gene are responsible for an autosomal recessive (AR) severe demyelinating form called CMT type 4A (OMIM 214400) [1,2], and axonal forms with [2], or without vocal cord paralysis (AR-CMT2K OMIM 607831) [3,4]. Mutations in this gene were found also in few family with a late-onset dominant form of CMT (AD-CMT2K) [5-7] and in patients presenting with a mixed phenotype including both axonal and demyelinating aspects [8].

AR-CMT patients with *GDAP1* mutations, show a severe phenotype characterized by a rapidly progressive weakness, usually beginning before the age of 3 years and eventually leading to inability to walk by the second or third decade. This phenotype can include vocal cord paresis, diaphragmatic paralysis, or kyphoscoliosis [9-12]. The dominant form is characterized by a much milder phenotype and onset during the second decade of life [5-7] with signs of axonal degeneration and demyelination at the histopathologic examination [6]. The majority of *GDAP1* mutations are associated with recessive CMT4A and with AR-CMT2K while only 4 were found in dominant CMT2K cases [5-7]. Few large *GDAP1* mutation screenings were conducted so far, and only in mixed population with both demyelinating and axonal AR-CMT patients [5,13]. *GDAP1* mutation frequency in axonal CMT (CMT2) is not known.

GDAP1 is a 358 amino acid protein belonging to a new subfamily of the glutathione-S-transferases (GST), a large family of enzymes sharing structural and functional similarities [1,2]. Most of these proteins are responsible for the metabolism of many compounds via conjugation with reduced glutathione. GDAP1 is located in the mitochondrial outer membrane, and unlike other GST enzymes, acts as mitochondrial fission factor regulating the mitochondrial network dynamics [14-17]. Recently, it was demonstrated that recessive *GDAP1* mutations are associated with reduced fission activity while dominant mutations impair mitochondrial fusion and cause mitochondrial damage [18].

We report the results of a *GDAP1* mutation analysis performed in 43 selected index patients affected by CMT2 (39) or an intermediate form of CMT (4), and negative for mutations in the genes most frequently involved in these forms. Of note, 27% of the dominant cases tested carry novel heterozygous mutations in this gene, an unexpected high percentage considering the low number of *GDAP1* heterozygous mutations reported so far. The genetic and clinical implications of these findings are discussed.

Patients and Methods

Forty three unrelated index patients from 43 families (total number of patients, 56) with a clinical diagnosis of axonal neuropathy were clinically and genetically analyzed. Thirty nine presented with a CMT2 phenotype with median nerve conduction velocities (MNCV) of 38m/s or greater while 4 presented an “intermediate CMT” phenotype with slightly reduced MNCV (from 25 to 38m/s). All showed severely reduced amplitudes of the compound motor and sensory nerve action potentials (or absent in some cases). Vocal cord paralysis with hoarseness or pyramidal signs were present in one and five sporadic cases respectively.

Twenty nine were sporadic (including the intermediate CMT) while 14 were familial cases, 11 with dominant segregation pattern of the disease while 3 were from recessive pedigrees. In one of the dominant families, P1086, the dominant inheritance is inferred by the clinical records of the proband's mother since she was not available to the authors for any clinical or genetic evaluation. Patients from recessive families and the proband of one of the dominant families (P227) had early onset (2-3 years of age) and severe disease status; almost all of them were wheelchair dependent by the age of 18 years. In all other patients, disease onset was in the age range of 7-20 years with the exception of 4 who had onset in the thirties, with a generally mild course with few exceptions. All patients were of Italian origin, 65% from the Northern part of the country, while 45% were from the South; all were in charge of the three Neurology services indicated in the affiliations. For all patients, mutations in *MPZ*, *MFN2*, *HSPB1*, *HSPB8*, *Cx32* and *NEFL* were previously excluded. *GARS*, *YARS* and *BSCL2* mutations were also excluded in all patients presenting with pyramidal features. The study was approved by the ethical committee of the E. Medea Scientific Institute. Blood specimens were obtained after informed consent. Genomic DNA from peripheral blood leukocytes was prepared with the standard high salt extraction method. *GDAP1* genomic region is available at the Human Genome Browser Gateway (<http://genome.ucsc.edu/cgi-bin/hgGateway>). All 6 exons of the *GDAP1* gene were amplified and mutation analysis was performed by sequencing of all exons with a Big Dye Terminator Sequencing Kit (version 3.1 Applied Biosystem Foster City, CA, USA). Mutation nomenclature is based on RefSeq NM_018972.2 [GenBank] (considering the A of the ATG as nt 1) and on Ref Seq NC_000008.9 for *GDAP1* cDNA and genomic sequences respectively, and follows the guidelines of the Human Genome Variation Society (<http://www.hgvs.org/mutnomen/>). Five hundred healthy individuals from the Italian population were used as controls. Missense changes were analysed with SIFT (<http://blocks.fhcrc.org/sift>) and Polyphen (<http://genetics.bwh.harvard.edu/pph/>) softwares.

Results

Mutation screening of *GDAP1* gene was performed in this series of 43 index patients, 39 with CMT2 and 4 with intermediate form of CMT with either familial or sporadic occurrence. The analysis revealed 3 novel heterozygous mutations in 3 families out of 11 with dominant inheritance of the disease (figure 1A). Clinical and electrophysiological data of the patients carrying *GDAP1* mutations, are summarized in Table 1 while a detailed description is provided as supplementary material (S1). Remarkably, in all three families symptoms onset was in the first decade in the probands while in the thirties (P227, P197) or twenties (P1086) in the carrier parents. Disease severity among the probands was also variable, with the most severe clinical picture and loss of

ambulation at 18 yrs, in the proband of family P227 who showed also the earliest onset (3 yrs). No pyramidal signs or vocal cord paralysis are present in any of the affected members of these families.

Table 1 Clinical and electrophysiological findings in three Italian families with heterozygous *GDAP1* mutations.

Patient ID	P197/ II-I	P197/ I-I	P227/II-I	P227/I-I	P1086/III-I	P1086/II-II
Age at onset/gender	8ys/F	35ys/F	3ys/F	30ys/F	7ys/F	20ys/F
Involved limb at onset	lower limbs	lower limbs	upper and lower limbs	lower limbs (EMG)	lower limbs	lower limbs
Age at last evaluation (years)	25 ys	49 ys	24 ys	53 ys	20 ys	20ys
Clinical involvement at last examination	foot paralysis, weakness small hand muscles++	no clinical signs, only EMG	foot paralysis and complete bilateral small hand muscle paralysis	lower limb weakness, mild vibration sense deficit	distal lower limbs severe muscular atrophy and upper limbs mild muscular hypotrophy; complete bilateral foot paralysis with a moderate weakness of the proximal muscles; mild weakness in the small hand muscles	slowly distal lower limbs weakness with associated mild hypotrophy
Walking ability	walks independently	walks independently	lost at 18 ys	walks independently	possible only with prothesis	walks independently
Others				dysthyroidism-arrhythmias	esophageal achalasia	
Motor nerve conduction velocity (m/s)						
ulnar (vn 46,5-72,6)	48,9	NA	47	NA	54,2	NA
tibial (39,8- 66,9)	27	45	25	42	absent	NA
Sensory nerve conduction velocity (m/s)						
median (36,5-65)	45,8	51	44,5	54	absent	NA
sural (35,6-63,6)	31,2	40	28,8	39	absent	NA
CMAP mV						
tibial nerve	3,5	6,8	3	5,6	absent	NA
peroneal nerve	0,3	10,9	1	7	absent	NA
SAP µV						
sural nerve	4,9	10	4	7	absent	NA
median nerve	4	9	3	9	absent	NA

CMAP: compound motor action potential; NA: not assessed; SAP: sensory action potential.

A nucleotide change c.678A>T in exon 5 was found in the proband of family P197, (figure 1A), leading to the missense mutation p.Arg226Ser. This mutation was inherited from the mother (P197 I-1) presenting only an abnormal electromyography (Table 1), while was absent in the healthy father and in 500 controls. The mutant residue is located within the GST domain of GDAP1 protein, in the α -helix 5 (α 5) within the α -motif II (figure 1B) and is highly conserved throughout evolution (figure 1S).

The proband of P1086 family carried a mutation in exon 1, c.101C>G leading to the missense change p.Ser34Cys (figure 1A). The mutant residue is located within the GST core region, two amino acids upstream the α -helix structure and is highly conserved (figure 1S). Only the healthy father was available for genetic analysis and was negative.

In family P227, the proband carried a heterozygous deletion in exon 1 (c.23delAG) leading to a frameshift and early protein interruption (p.Gly10GlufsX14) (figure 1A). A nucleotide change in exon 6 c.805G>A, was also detected causing the p.Gly269Arg substitution. While the deletion is inherited from the affected mother, the missense variation was detected in the unaffected father and in two out of 500 controls (allele freq 0.2).

Analysis of all missense changes found with SIFT and Polyphen softwares indicates that p.Arg226Ser and p.Ser34Cys are both predicted to damage protein structure and affect protein function (figure 1A). The p.Gly269Arg substitution is instead compatible with normal protein function for both programs. Its location, far from the α -helix structure, and the segregation pattern both favour the hypothesis of p.Gly269Arg being a rare missense variant.

Discussion

We report the results of a *GDAP1* mutations screening performed in 39 CMT2 and 4 intermediate CMT patients, with either sporadic or familial occurrence. While no mutations were observed in sporadic or recessive patients, 3 novel heterozygous *GDAP1* mutations were found in 3 out of 11 dominant families. This result leads to a *GDAP1* mutations frequency of 27% in AD-CMT2, a figure so far unreported for *GDAP1*. A similar frequency is known for *MFN2* which is responsible for 17-33% of dominant cases with CMT2 [19,20]. Although the *GDAP1* mutation frequency we observed needs to be confirmed in larger series of patients, the results obtained suggest that *GDAP1* involvement in AD-CMT2 might be higher than expected. In fact only four heterozygous mutations were reported so far in this form [5-7]. Ethnic origin and geographical distribution of the patients here tested *per se*, hardly account for the high *GDAP1* mutation frequency observed. Our families carrying a *GDAP1* mutation are all from different Italian regions. It is more likely that the

preliminary exclusion of mutations in the genes more frequently involved in CMT2 in the patients here tested might have partly contributed to this unexpected result. The lack of mutations observed in our group of sporadic patients compared to a known frequency of 5,6% [5] might be explained both by the lower sample size (29 vs 106) and by the different types of patients samples in the two studies: only axonal CMT patients in this study compared with mixed axonal and demyelinating patients in the previous one.

The AD-CMT2K phenotype described in previous reports was mild with disease onset in the second or third decade and a mild course. The clinical picture emerging from the families here described partly overlaps to that phenotype with a marked intra- and inter-familial variability, mostly in disease severity and symptoms onset. Indeed, in all three families symptoms onset was in the first decade in the probands while in the thirties (P227, P197) or twenties (P1086) in the carrier parents. In P227 II-1, the age at disease onset is very early (3 years). Unexpectedly in the carrier mother (P227 I-1), the disease is manifesting in a very mild form with only subclinical signs in the thirties, then evolving to mild clinical symptoms only in the late forties. Disease severity is also apparently directly correlated with the age at disease onset, with the most severe clinical picture (loss of ambulation at 18 yrs) displayed by the patient with the earliest onset (P227 II-1).

The majority of the *GDAP1* mutations associated with AD-CMT2K, affect the GST domain, the hallmark of the glutathione S-transferases (GSTs), enzymes that have a role in the detoxification of cells. *GDAP1* belongs to a new subfamily of GSTs [2,21] for which no GST activity was proved so far [21]. It was instead demonstrated to regulate mitochondrial network by promoting mitochondrial fission rather than detoxifying reactive oxygen species [14]. The GST domain lies within the cytoplasmic N-terminal region with two motifs: motif I that corresponds to the glutathione binding site (aa 26-119) in which the aminoacid residues have a secondary structure $\beta\alpha\beta\alpha\beta\alpha$, and the α -motif II (aa 210-287) associated with presumed xenobiotic and cytotoxic activities [2]. All known mutation associated with the AD-CMT2K phenotype fall within the two motifs with the exception of one p.Thr157Pro, falling in the region between the two. With regard to the novel heterozygous mutations here reported, c.23delAG is truncating at the N-terminus just upstream the GST domain, p.Ser34Cys lies within the GST domain just upstream the α -helix 1 while p.Arg226Ser falls within the α -helix 5. The two novel missense changes are both predicted to have a damaging effect by using two prediction softwares (figure 1A) and both affect evolutionary conserved residues (figure 1S). In particular Serine 34 residue represents the catalytically active residue positioned at the N-terminal of α -1 in the thioredoxin domain, which is shared by all enzymatically active GSTs [22]. The mutant cysteine residue (C34) is shared only by the omega class of GSTs [21] that had been

shown to have poor GST activity with common substrates being rather involved in Ca^{2+} -release modulation through ryanodine receptors (RYR1) [23].

Although no GST activity was demonstrated so far for GDAP1 [22], based on the mutations results in AD-CMT2K patients reported here and elsewhere, GST domain turns out to be a frequent target for mutations in AD-CMT2K. This suggests that whatever is the function associated with this domain, this has to be relevant in the pathogenesis of AD-CMT2K disease. In addition, targeting of all mutations to the α -helices of the GST domain suggests that the α -helices integrity is also a strong structural requirement for GDAP1 activity to be performed.

Three of the four known mutations associated with AD-CMT2K, p.Arg210Trp, p.Thr157Pro, p.Gln218Glu, were in fact demonstrated to impair mitochondrial fusion leading to elevated ROS levels and to increase susceptibility to apoptosis [18]. According to the authors' predictions [18], an analogous deleterious gain of function can be also postulated for other dominant missense mutations with shared location such those here reported. By contrast, the heterozygous two-bp-deletion described here, leading to early protein truncation (p.Gly10GlufsX14) and therefore to a likely loss of function, is in fact associated with a very mild phenotype (see the carrier mother in family P227). The additional presence of the heterozygous variant p.Gly269Arg (still in the α -motif II of the GST domain) in the proband P227 II-1, associates with a more dramatic effect on clinical phenotype (Table 1). Since this missense variant by itself is not associated with a pathological phenotype, p.Gly269Arg may have a role as phenotype modulator with an allele frequency in the Italian population of 0,2% .

From a different point of view, overall the results obtained provide some more clues of clinical and diagnostic value for AD-CMT2K, mostly underlying the variable penetrance and different clinical expressivity associated with all mutations identified. Although with the limitation due to the relatively small sample size, these data also suggest the opportunity to screen *GDAP1* gene in autosomal dominant patients with CMT2, negative for mutations in *MFN2* gene.

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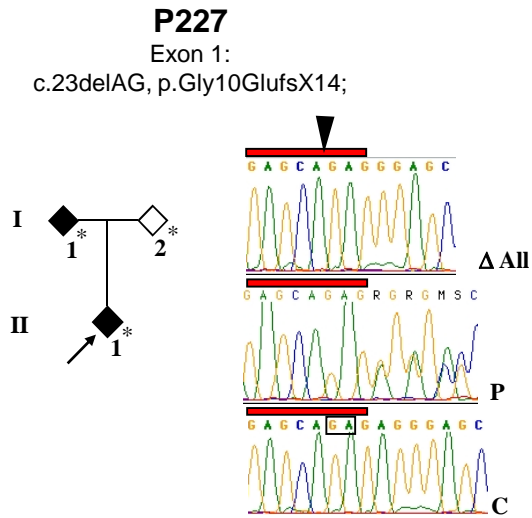
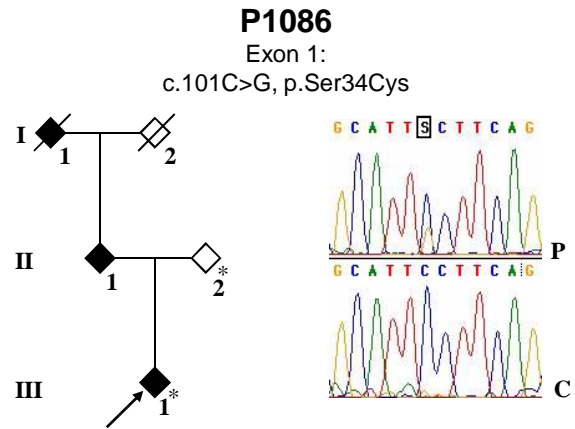
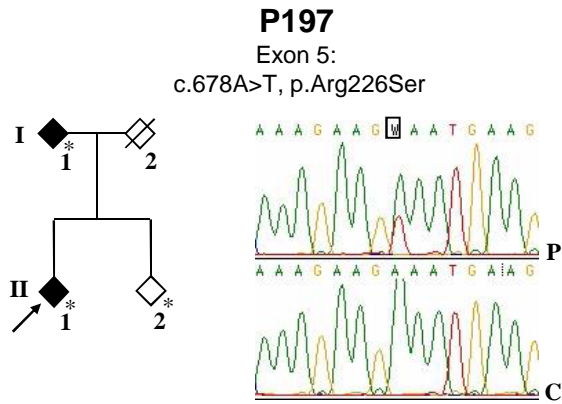
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Figure Legend

Figure 1: A. The pedigree of families with *GDAP1* mutations; diamond symbols are for anonymity reasons, stars and arrows indicate subjects whose DNA is available and probands respectively. Electropherograms show the mutant sequences and the corresponding controls. Boxed sequences are either the mutant nucleotide (P197, P1086) or the deleted sequence (P227). The arrowhead in pedigree P227 shows the deletion site; Δ -allele indicates the subcloned deleted allele. On the right SIFT and Polyphen software's prediction are summarized. With SIFT program the score ranges from 0 to 1, where 0 is damaging and 1 is neutral. Polyphen's score ranges from 0 to a positive number, where 0 is neutral and a high positive number is damaging. For the exonic variant p.Gly269Arg the SIFT's protein prediction is 0.11 (tolerated); Polyphen's prediction is 1.501 (possibly damaging).

B. Schematic representation of *GDAP1* protein domains with the heterozygous mutations identified; the novel ones are circled. The dashed bars below indicate the two motifs constituting the glutathione S-transferases domain. HD1: hydrophobic domain 1; TMD:transmembrane domain.

A



Patient ID	Mutation	Protein		Other exonic variants
		POLYPHEN	SIFT	
P197 II-I/I-I	c.678A>T p.Arg226Ser	1.927 (possibly damaging)	0.01 (affect protein function)	
P1086 III-I/II-I/I-I	c.101C>G p.Ser34Cys	1.924 (possibly damaging)	0.00 (affect protein function)	
P227 II-I/I-I	c.23delAG p.Gly10GlufsX			p.Gly269Arg

B

