Neurogenetics (2010) 11:357–366
DOI 10.1007/s10048-010-0237-6

ORIGINAL ARTICLE

L239F founder mutation in *GDAP1* is associated with a mild Charcot–Marie–Tooth type 4C4 (CMT4C4) phenotype

Dagmara Kabzińska · Halina Strugalska-Cynowska · Anna Kostera-Pruszczyk · Barbara Ryniewicz · Renata Posmyk · Alina Midro · Pavel Seeman · Lucia Báranková · Magdalena Zimoń · Jonathan Baets · Vincent Timmerman · Velina Guergueltcheva · Ivailo Tournev · Stayko Sarafov · Peter De Jonghe · Albena Jordanova · Irena Hausmanowa-Petrusewicz · Andrzej Kochański

Received: 4 December 2009 / Accepted: 18 February 2010 / Published online: 16 March 2010

Abstract Over 40 mutations in the *GDAP1* gene have been shown to segregate with Charcot–Marie–Tooth disease (CMT). Among these, only two mutations, i.e., S194X and Q163X have been reported in a sufficient number of CMT families to allow for the construction of reliable phenotype–genotype correlations. Both the S194X and Q163X mutations have been shown to segregate with an early-onset and severe neuropathy resulting in loss of ambulance at the beginning of the second decade of life. In this study, we identified the L239F mutation in the *GDAP1* gene in one Bulgarian and five Polish families. We hypothesized that the L239F mutation may result from a founder effect in the European population since this mutation has previously been reported in Belgian, Czech, and Polish patients. In fact, we detected a common disease-associated haplotype within the 8q13-q21 region in the Polish, German, Italian, Czech, and Bulgarian CMT families. Like the previously detected "regional" S194X and Q163X mutations, respectively present in Maghreb countries and in patients of Spanish descent, the L239F mutation seems to be the most common *GDAP1* pathogenic variant in the Central and Eastern European population. Given the likely presence of a common ancestor harboring the L239F

Halina Strugalska-Cynowska is already deceased.

D. Kabzińska · H. Strugalska-Cynowska · I. Hausmanowa-Petrusewicz · A. Kochański (✉)
Neuromuscular Unit,
Mossakowski Medical Research Centre,
Polish Academy of Sciences,
Pawińskiego 5,
02-106 Warsaw, Poland
e-mail: andko@cmdik.pan.pl

A. Kostera-Pruszczyk · B. Ryniewicz
Department of Neurology, Medical University of Warsaw,
Warsaw, Poland

R. Posmyk · A. Midro
Department of Clinical Genetics,
Medical University of Białystok,
Bialystok, Poland

P. Seeman · L. Báranková
Department of Child Neurology, 2nd School of Medicine,
Charles University in Prague,
Prague, Czech Republic

M. Zimoń · J. Baets · P. De Jonghe · A. Jordanova
Neurogenetics Group, VIB Department of Molecular Genetics,
University of Antwerp,
Antwerp, Belgium

M. Zimoń · J. Baets · P. De Jonghe · A. Jordanova
Laboratory of Neurogenetics, Institute Born-Bunge,
University of Antwerp,
Antwerp, Belgium

J. Baets · P. De Jonghe
Division of Neurology, University Hospital Antwerpen (UZA),
Antwerp, Belgium

V. Timmerman
Peripheral Neuropathy Group,
VIB Department of Molecular Genetics, University of Antwerp,
Antwerp, Belgium

V. Guergueltcheva · I. Tournev · S. Sarafov
Department of Neurology, Sofia Medical University,
Sofia, Bulgaria

mutation, we decided to compare the phenotypes of the CMT (L239F) patients collected in this study with those of previously reported cases. In contrast to CMT4A caused by the S194X and Q163X mutations, the CMT phenotype resulting from the L239F substitution represents a milder clinical entity with a long-preserved period of ambulance at least until the end of the second decade of life.

Keywords Phenotype–genotype correlations in CMT4A disease · Founder effect · The L239F mutation in the *GDAP1* gene

Introduction

Charcot–Marie–Tooth type 4C4 disease (CMT4C4) seems to be the most common peripheral neuropathy with an autosomal recessive mode of inheritance.

The recessive axonal CMT4C4 (OMIM #607776) and demyelinating CMT4A (OMIM #214400) forms have been mapped to the 8q13-21 region in a series of consanguineous families [6].

In 2001, Cuesta and Baxter independently reported mutations in the gene coding for the *ganglioside differentiation-associated protein 1* (*GDAP1*).The patients were affected with an early onset, severe demyelinating or axonal neuropathy [5, 13].

Recently, a set of *GDAP1* gene mutations have been reported that behave as an autosomal dominant trait and have been designated as dominant axonal CMT2K (OMIM #607831) [11, 12].

In the majority of cases, the CMT4C4 phenotype is characterized by onset at an early age, usually before the age of 5 years, and a severe clinical course often leading to wheelchair-bound status even in childhood [16].

Due to the small number of patients/families harboring the same *GDAP1* mutation phenotype–genotype correlations are difficult to construct in CMT4C4.

An exception to this rule is presented by two recurrent *GDAP1* mutations, i.e., Q163X and S194X in patients originating from Maghreb countries (S194X) or of Spanish origin (Q163X). It was recently shown that these two *GDAP1* gene mutations result from the founder effect [5, 8, 12, 13].

In the study described here, we aimed to characterize the CMT4C4 phenotype which is associated with the third recurrent *GDAP1* gene mutation, i.e., L239F, which we proved to have a common origin in the European population.

Material and methods

The study was carried out with Approval No. 120/08 of the Bioethics Committee at Medical University of Warsaw. Additionally, all patients and healthy family members taking part in this study signed an informed consent form allowing us to perform analysis of their DNA.

For this study, 38 CMT families were enrolled among the more than 500 pedigrees archived at the Neuromuscular Unit, Mossakowski Medical Research Centre of Polish Academy of Sciences.

The entry criteria applied were: (1) a recessive mode of inheritance, (2) no data suggesting a dominant mode of inheritance, (3) axonal form of CMT revealed by nerve conduction study, and (4) exclusion of *PMP22* gene duplication/deletion.

DNA was isolated from blood samples of 50 CMT-affected patients representing 38 families.

Initially, quantitative Q-PCR analysis was performed in these patients in order to rule out *PMP22* deletion/duplication defects.

For the 8q13-q21 haplotype analysis, we obtained DNA samples from CMT4C4 affected individuals from the laboratories of Prof. P. De Jonghe (Neurogenetics Group, VIB Department of Molecular Genetics, University of Antwerp, Antwerp, Belgium) and Assoc. Prof. P. Seeman (Department of Child Neurology, 2nd School of Medicine, Charles University, Prague, Czech Republic). The clinical and electrophysiological characteristics of these patients have been previously reported [4, 16].

Clinical and electrophysiological characteristics of a Bulgarian (CMT Ant 1) family were provided by Drs. Guergueltcheva, Tournev, and Sarafov of the Department of Neurology, Sofia Medical University, Bulgaria.

Molecular analysis of the CMT Ant 1 family was performed by the Neurogenetics Group, VIB Department of Molecular Genetics, University of Antwerp, Antwerp, Belgium.

Real time PCR (Q-PCR)

Q-PCR analysis was carried out in line with the procedure described previously by Aarskog and Vedeler [1].

In brief, the TaqMan probe for the *PMP22* gene contains a flurophore 5′FAM as a reporter and 3′TAMRA as a quencher, while the TaqMan probe for the human serum albumin gene contains 5′VIC as a reporter and 3′TAMRA as a quencher.

PCR analysis was performed using the 7500 Real Time PCR System (Applied Biosystems). Relative *PMP22* copy number was calculated using computer software (7500 System Software) supplied by Applied Biosystems.

Single-strand conformation polymorphism analysis/heteroduplex analysis and sequencing of the *GDAP1* gene

The coding sequence of the *GDAP1* gene was amplified by means of PCR with previously reported primers [13]. The *GDAP1* gene sequence was screened for the presence of

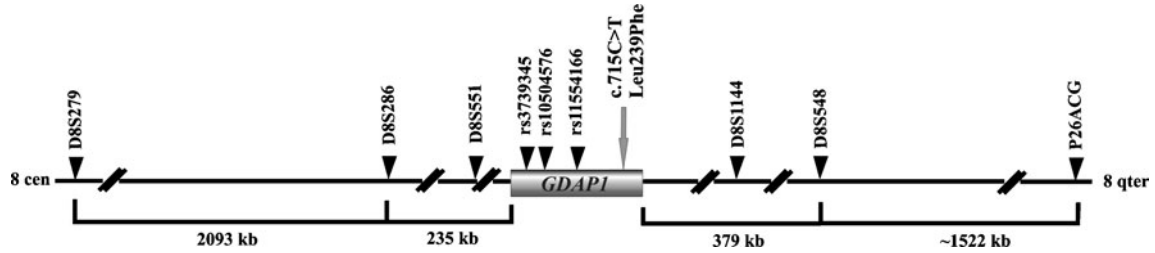

Fig. 1 Physical map of the CMT4C4/4A/2K *locus.* The *GDAP1* gene is indicated as a *box*. The microsatellite markers and SNP markers are indicated at the *top of the figure*

DNA variants using two separate approaches, i.e., single-strand conformation polymorphism analysis (SSCP) and heteroduplex analysis (HA).

In the SSCP and HA analyses, PCR products were separated on 9% acrylamide gels (37.5:1 acrylamide/bisacrylamide). The gels were silver stained, dried, analyzed, and documented.

For the PCR fragments showing an altered migration pattern in comparison with the wild-type allele pattern, direct sequencing was carried out using a BigDyeTM Terminator Version 3.1 Ready reaction Cycle Sequencing kit on the ABI 3730/xl Genetic analyzer (Applied Biosystems).

The *GDAP1* gene sequence was analyzed by comparison with reference sequences NM-018972.2 variant1 mRNA reference and NC-000008.9 (for the genomic DNA).

Haplotype analysis at the 8q13-q21 *locus*

Genotyping was performed using six STR markers (D8S279, D8S548, D8S551, D8S286, D8S1144, P26ACG) and three intragenic SNP markers (rs11554166, rs3739345, rs10504576; Fig. 1) encompassing the 8q13-q21 *locus*. The STR and SNP markers were selected on the basis of previous reports [4, 7, 17].

DNA was amplified using fluorescent-labeled primers in a PCR reaction volume of 12.5 μl containing 2 pmol of each primer, 2.5 mM of each dNTP; 1.25 μl 10× PCR buffer, 19 mM $MgCl_2$, and 0.5 U TaqDNA polymerase Fermentas.

PCR for STR markers was performed under the following conditions: for 1 min at 95°C, 45 s at 49–52°C depending on the primer pair and 1 min at 72°C for 11 cycles and 1 min at 90°C, 45 s at 49-52°C, 1 min at 72°C for 23 cycles, final elongation 30 min at 72°C. The PCR conditions for SNP markers were set as follows: 3 min initial denaturation at 95°C, 30 cycles of 45 s denaturation at 95°C, 45 s annealing at touchdown 63–48°C, 1 min elongation at 72°C and ten cycles of 45 s at 95°C, 45 s at 48°C and 1 min at 72°C, final elongation 7 min at 72°C.

The diluted PCR products for STR markers were loaded with GeneScan 500LIZ size standard in an ABI 3730/xL automated DNA sequencer.

Table 1 Clinical and genetic data for different index patients

Patient/family no.	Familial/sporadic	Age at onset (year)	Age (year) at last examination	Initial symptoms	Additional features	Ambulancy	Clinical diagnosis	*GDAP1* mutation
E (III:3)	Sporadic	2	25	Unsteady gait	Hand tremor, hoarseness	+	CMT2	Leu239Phe/Arg273Gly
A (III:12)	Familial	4	35	Club feet	Finger tremor	Wheelchair-bound since age 34	CMT2	Leu239Phe/Leu239Phe
A (III:20)	Familial	Early childhood	22	Unsteady gait	−	Wheelchair-bound since age 18	CMT2	Leu239Phe/Leu239Phe
C (IV:3)	Familial	3	8	Unsteady gait	−	+	CMT2	Leu239Phe/Arg282Cys
C (IV:4)	Familial	2	5	Toe walking	−	+	CMT2	Leu239Phe/Arg282Cys
B (III:3)	Sporadic	5	8	Gait abnormality	Lumbar lordosis	+	CMT2	Leu239Phe/Leu239Phe
D (III:7)	Familial	5	8	Frequent falls	−	+	CMT2	Leu239Phe/Met116Thr
D(III:8)	Familial	10	12	Frequent falls	−	+	CMT2	Leu239Phe/Met116Thr
CMT Ant (1.1)	Familial	4	23	Club feet	−	+	CMT2	Leu239Phe/Asn227Asp
CMT Ant (1.2)	Familial	10	20	Club feet, gait abnormality	−	+	CMT2	Leu239Phe/Asn227Asp

All have progressive distal muscle weakness and wasting of the upper and lower limbs. Patients are denoted in line with positions in pedigrees (for details see Fig. 2)

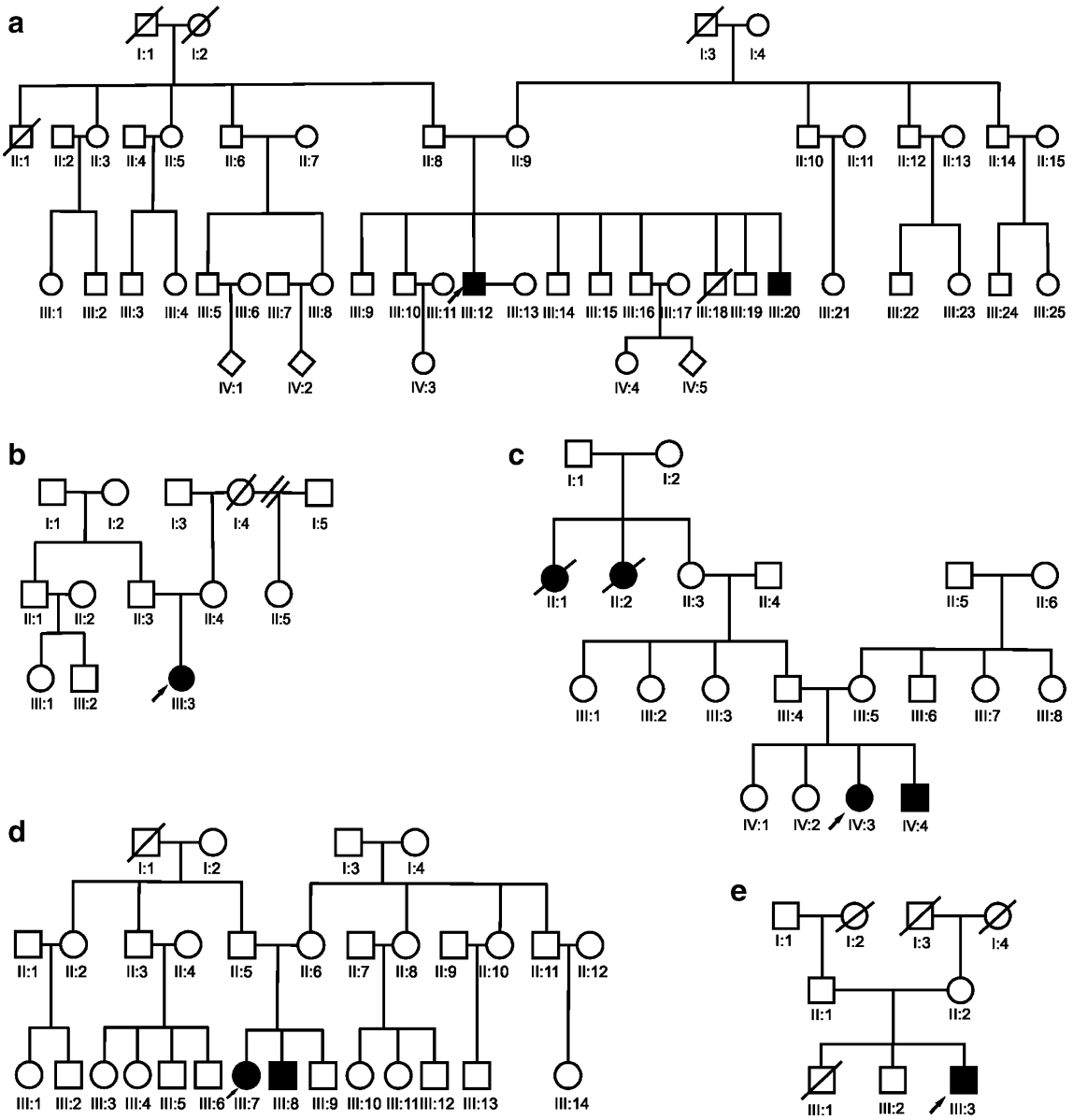

Fig. 2 The pedigrees of the CMT4A families in which L239F mutation was identified in the *GDAP1* gene. The probands are indicated with an *arrow*. The females are denoted by *circles* and males by *squares*. A *filled symbol* denotes a CMT-affected patient. Deceased individuals are marked with a *diagonal line*

The RFLP-PCR analysis for SNPs (rs1154166; rs 3739345 and rs 10504576) was performed with *Dde*I, *Hph*I, and *Acc*I resctriction endonucleases, respectively, in line with the manufacturer's instructions.

The data for STR markers were collected and analyzed using the Peakscan software from Applied Biosystems. Haplotypes for the 8q13-21 *locus* were constructed manually.

Allele frequencies were established by testing 50 healthy, unrelated individuals.

The presence of the L239F mutation in the controls was excluded by the RFLP-PCR approach with the *Mwo* I enzyme in line with the instructions of the manufacturer.

Estimation of the L239F mutation age was performed using the DMLE+ linkage disequilibrium mapping software (available at http://dmle.org).

The program parameter setting we used were: number of chromosomes in the disease sample = 36; number of marker *loci*=4; number of haplotypes in the normal (base) population for 60 healthy controls; map distance = 0.0, 0.28924, 0.38824, 1.57671; mutation location = 0.3582; population growth rate = 0.0055; proportion of population sampled = 0.0009; burn-in interactions = 1×10^6; interactions = 1×10^6; mutation age—random; mutation age boundaries from 0 to 50.000 generations.

Results

The clinical characteristics of the examined patients are presented in Table 1.

The majority (eight out of ten) of the reported patients represent familial CMT cases. An autosomal recessive trait of inheritance was evident in four families; while in two, sporadic cases were observed (Fig. 2).

The ages of examined patients at the last examination ranged from 5 to 35 years. Six patients were male, four patients were female. No delayed motor milestones were recorded in the examined patients. All the CMT4A-affected patients revealed first symptoms in childhood at ages ranging from 2 to 10 years. The heterozygous carriers of the L239F mutation did not display any CMT symptoms.

Initial symptoms varied from unsteady gait, toe walking, to club feet. Affected children were ambulant, but two affected brothers became wheelchair-bound at the age of 18 and 34 years. The 25-year-old patient [E(III:3)] harboring the heterozygous L239F/R273G mutation was still ambulant. Severe progressive distal muscle wasting in the upper and lower limbs was observed in six patients, while sensory disturbances were to be noted in all of them.

In two patients, finger tremor was observed, while one patient had lumbar lordosis. No vocal cord or phrenic nerve palsy was detected.

Results for the electrophysiological examinations performed on nine patients are given in Table 2.

CMAP amplitudes in the median and tibial nerves were reduced in all patients examined. In five of them, no motor response was obtained from the peroneal nerve because of severe muscle atrophy. SNAPs were obtained from the median nerve in three patients but they were severely reduced. In two patients, median SNAPs were not recordable. In four examined patients, ulnar SNAPs were not recorded. In the sural nerve examination SNAPs were not obtainable in six patients and greatly reduced in one.

The median MNCVs in eight patients were within the normal range. In the two Bulgarian patients (CMT Ant 1.1, CMT Ant 1.2), median MNCV and CMAPs displayed moderate reduction.

Overall, there was axonal sensory–motor neuropathy, with different degree of involvement in studied patients.

The results of analysis of the *GDAP1* gene sequence

To exclude the duplication and deletion of the *PMP22* gene, quantitative Q-PCR analysis was performed in all affected individuals. Given the reference range of 0.700 to 1.090, the CMT4C4 affected patients had *PMP22* gene dosage between 0.721 and 1.071.

In the SSCP and HA approaches, an altered migration pattern was observed in seven PCR products corresponding with the exon 6 of the *GDAP1* gene.

In three patients, direct sequencing of the *GDAP1* gene (performed in the forward and reverse orientation) revealed

Table 2 Electrophysiological data for respective index patients

Family/Patient	Median nerve / motor nerve conduction		Peroneal nerve/ motor nerve conduction		Tibial nerve/ motor nerve conduction		Median nerve/ sensory nerve conduction		Ulnar nerve/ sensory nerve conduction		Sural nerve	
	CMAP (mV)	MNCV (m/s)	CMAP (mV)	MNCV (m/s)	CMAP (mV)	MNCV (m/s)	SNAP (μV)	SNCV (m/s)	SNAP (μV)	SNCV (m/s)	SNAP (μV)	SNCV (m/s)
C(III:3)	4.1	63.2	n.r.		1.7	47.2	2.2	35.7	n.r.		n.r.	
C(IV:4)	0.9	55.0	0.7	55.0	0.4	46.5	3.6	49.5	n.d.		n.r.	
B (III:3)	4.2	51.7	3.1	75	0.4	40	5.2	41.1	n.d.		6.7	50
D (III:7)	3.8	59.7	n.r.	n.r.	9.2	52.6	n.r.	n.r.	n.r.		n.r.	
D (III:8)	n.d.	n.d.	n.r.	n.r.	0.50	38.7	1.76	39.7	n.r.		n.r.	
E(III:3)	0.1	52.1	n.r.	n.r.	n.d.	n.d.	n.r.	41.0	n.d.		n.d.	
CMT Ant (1.1)	3.0	37.9	0.57	37.2	0.89	33.2	3.7	n.d.	n.r.		n.r.	
CMT Ant (1.2)	4.3	43.9	n.d.	n.d.	0.29	27.8	5.8	n.d.	23.7		n.r.	
A (III:12)	4.3	55.0	n.r.	n.r.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	

Normal values: median MNCV=59.11±4.08 m/s; median CMAP=8.8±2.91 mV; peroneal MNCV=50.24±5.85 m/s; peroneal CMAP=6.1±2.59 mV; tibial MNCV=50.24±3.23 m/s; tibial CMAP=11.6±4.3, median SNCV=62.37±7.3 m/s; median SNAP=12.29±7.05 μV; ulnar SNCV=60.8±5.07, ulnar SNAP=11.12±5.3 μV; sural SNAP=17.46±5.44 μV; sural SNCV=53.2±4.19 m/s

MNCV motor nerve conduction velocity, *SNCV* sensory nerve conduction velocity, *CMAP* compound muscle action potential, *SNAP* sensory nerve action potential, *n.d.* not done, *n.r.* not recordable

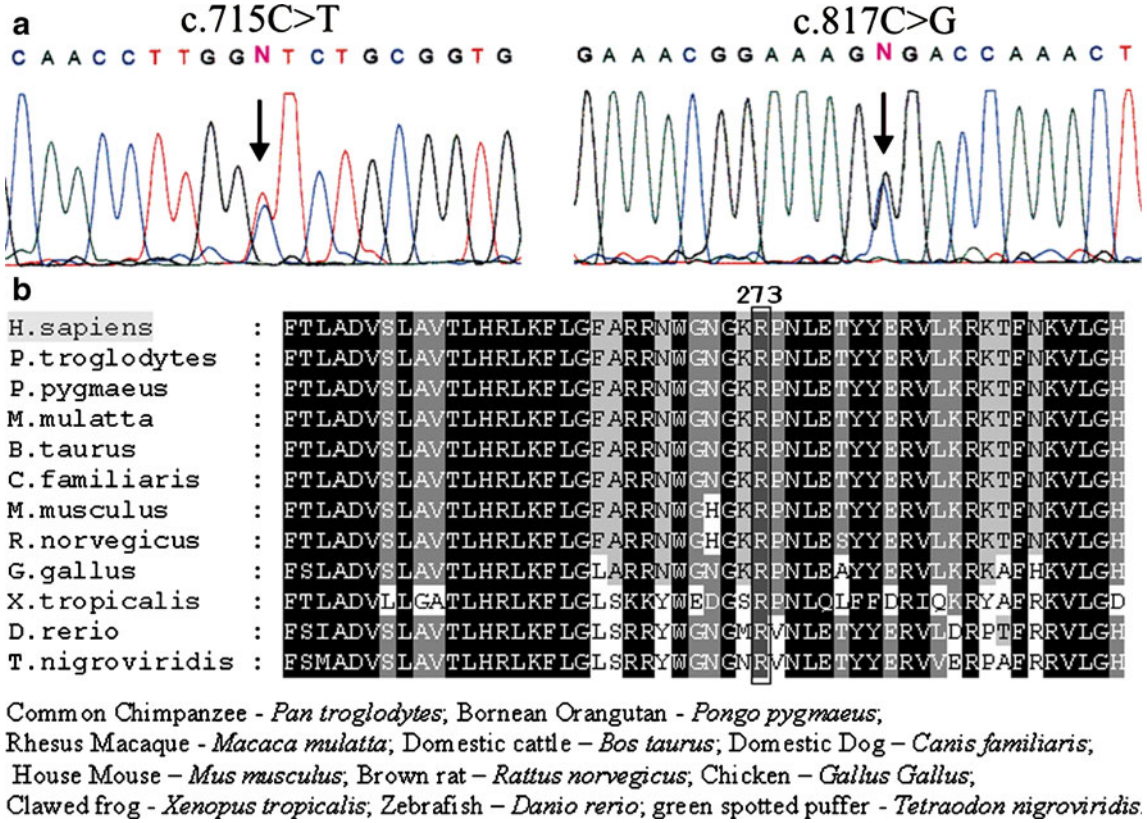

Fig. 3 **a** Identification of the R273G mutation in the *GDAP1* gene. A direct sequencing analysis revealed a heterozygous C to G transversion (*arrow*) at the nucleotide 817 of the *GDAP1* gene resulting (by conceptual translation) in the R273G missense mutation. **b** Conservation of the R273G mutation found in the *GDAP1* gene across various species. Amino acid number 1 is the first methionine of the open reading frame of the *GDAP1* gene. The Arg amino acid indicated in the codon 273 is conserved across 11 species. *Homo sapiens* human being, *Pan troglodytes* common chimpanzee, *Pongo pygmaeus* Bornean Orangutan, *Macaca mullata* rhesus Macaque, *Bos taurus* cattle, *Canis familiaris* domestic dog, *Rattus norvegicus* brown rat, *Gallus gallus* chicken, *Xenopus tropicalis* clawed frog, *Danio rerio* Zebra fish, *Tetraodon nigroviridis* green spotted puffer

a homozygous C to T transition at the nucleotide 715, this resulting (by conceptual translation) in a leucine to phenylalanine substitution at codon 239 of the *GDAP1* gene (L239F).

In five healthy family members, a heterozygous L239F mutation was also identified.

Two patients showed a compound heterozygosity for L239F/R282C mutations in the *GDAP1* gene. One patient, in turn, was a compound heterozygote for L239F and a novel R273G mutation (Fig. 3).

Finally, in one patient [B(III:3)], there was a compound heterozygous L239F/M116T mutation in the *GDAP1* gene.

The results of haplotype analysis at the 8q13-q21 *locus*

In order to determine the common ancestral origin of the L239F mutation we searched for shared disease-associated haplotypes.

The haplotype analysis was performed on DNA samples obtained from patients of Polish, Czech, German, Italian, and Bulgarian origin.

We constructed haplotypes using six STR markers and three SNP markers located in the 8q13-21 region.

Interestingly, all the CMT4A-affected patients (four Polish and two Czech families) harboring the homozygous L239F mutation in the *GDAP1* gene showed the presence of a common haplotype: -229-271-T-A-G-150-233- (Table 3).

The distance between the D8S286 and D8S548 covers a 614 kb region in the 8q13-q21 locus.

The heterozygous carriers of the L239F mutation in the *GDAP1* gene derived from patients of Polish, Czech, German and Bulgarian origin also showed a common haplotype of -229-271-T-A-G-150-233-. In three individuals, the 269, 269, and 273 bp alleles were found with D8S551 and in four subjects 146, 146, 154, and 158 bp alleles with D8S1144.

Among the control chromosomes (100 in all), the vast majority showed the 233-bp allele with D8S286 and the remaining STR and SNP markers were represented by a random spectrum of alleles.

At the most distal D8S279 (centromeric) and P26ACG (telomeric) markers, a wide spectrum of alleles were

Table 3 *GDAP1* haplotypes cosegregating with the L239F mutation in a homozygous and a heterozygous state. The families and chromosome number have been indicated in the first and second column, respectively

		5′flanking markers			*GDAP1* SNP			3′flanking markers			
Family	Chromosome no.	D8S279	D8S286	D8S551	rs3739345 A/T	rs10504576 A/G	rs11554166 c.507 G/T	D8S1144	D8S548	P26ACG	Ethnic origin
A(III.12)	A	248	229	271	T	A	G	150	233	140	Polish
	B	248	229	271	T	A	G	146	233	136	
A(III.20)	C	248	229	271	T	A	G	150	233	140	Polish
	D	248	229	271	T	A	G	146	233	140	
A(II.8)	E	248	229	271	T	A	G	150	233	140	Polish
A(III.16)	F	248	229	271	T	A	G	150	233	140	Polish
A(II.10)	G	248	229	271	T	A	G	150	233	140	Polish
A(II.9)	H	248	229	271	T	A	G	150	233	140	Polish
B(III.3)	I	225	229	271	T	A	G	150	233	140	Polish
	J	227	229	271	T	A	G	150	233	142	
CMTWar1	K	231	229	271	T	A	G	150	233	140	Polish
	L	227	229	271	T	A	G	158	233	142	
C(IV.3)	M	227	229	271	T	A	G	150	233	140	Polish
C(IV.4)	N	227	229	271	T	A	G	150	233	140	Polish
E(III.3)	O	225	229	273	T	A	G	150	233	136/142	Polish
E(II.1)	P	225	229	273	T	A	G	150	233	136/142	Polish
D(III.7)	Q	231	229	271	T	A	G	150	233	140	Polish
D(II.6)	R	231	229	271	T	A	G	150	233	140	Polish
D(III.8)	S	231	229	271	T	A	G	150	233	140	Polish
D(III.9)	T	231	229	271	T	A	G	150	233	140	Polish
CMTWar2.1	U	248	229	271	T	A	G	150	233	140	Polish
CMTWar2.2	W	248	229	271	T	A	G	150	233	140	Polish
CMTPraque1	V	248	229	271	T	A	G	150	233	136	Moravia
	X	244	229	271	T	A	G	150	233	142	
CMTPraque2	Y	225	229	271	T	A	G	150	233	140	Moravia
	Z	244	229	271	T	A	G	154	233	136	
CMTPraque3	AA	248	229	269	T	A	G	150	233	140	Moravia
CMTPraque4	AB	248	229	271	T	A	G	150	233	142	West Bohemia
CMTPraque5	AC	248	229	269	T	A	G	150	233	142	Moravia
CMTPraque6	AD	248	229	271	T	A	G	150	229/231	140/142	Moravia
CMTAnt1.1	AE	225/231	229	271	T	A	G	150	233	136/142	Bulgarian
CMTAnt1.2	AF	225/231	229	271	T	A	G	150	233	136/142	Bulgarian
CMTAnt2.1	AG	248	229	271	T	A	G	150	233	140	German/

Table 3 (continued)

Family	Chromosome no.	5′flanking markers			*GDAP1* SNP			3′flanking markers			
		D8S279	D8S286	D8S551	rs3739345 A/T	rs10504576 A/G	rs11554166 c.507 G/T	D8S1144	D8S548	P26ACG	Ethnic origin
CMTAnt2.2	AH	248	229	271	T	A	G	150	233	140	Italian German/ Italian
CMTAnt2.3	AI	248	229	271	T	A	G	150	233	140	German
Marker allele frequencies in controls			0.186	0.000	0.520	0.608	0.520	0.078	0.363		

The ethnic origin of the families is shown in the last column. The families reported for a first time in this study have been denoted according to the position in the pedigree (Fig. 2)

The families reported in the previous studies in which haplotype analysis was performed are denoted with the following abbreviations: *CMT War 1* DNA sample from the CMT family, collected in the Neuromuscular Unit, MRC, Warsaw; *CMT Ant 2* DNA sample from the family, collected in the Peripheral Neuropathy Group at the University of Antwerp, *CMT Prague 1* DNA sample from the family, collected in the Department of Child Neurology, 2nd School of Medicine, Charles University, Prague

identified in both homozygous and heterozygous L239F carriers and in controls.

Due to the presence of a common -229-271-T-A-G-150-233- haplotype from the homozygous and heterozygous carriers of the L239F mutation, and the absence of this haplotype in the control chromosomes of the ethnically matched origin, we postulate that all our CMT4C4 patients with L239F mutation are with common origin as a result of an ancestral founder effect.

By using DMLE+ software, we estimated the age of the L239F mutation to be 22,000 years.

Discussion

In contrast to CMT1A disease caused by a tandem duplication in 17p11.2-p12 for which hundreds of patients have been characterized in detail, CMT4C4 disease is a clinical entity in which genotype–phenotype correlations are extremely hard to describe.

In fact, the vast majority of *GDAP1* causative mutations have been reported in single families, this complicating the obtainment of genotype–phenotype correlations based on a reliable number of patients with the same *GDAP1* mutation.

Only two *GDAP1* gene mutations (i.e., S194X and Q163X) have been reported in a few CMT4 families.

The S194X mutation was detected in Morocco, Algeria, and Tunisia as well as a Belgian family of Moroccan ancestry [5, 7, 9, 12, 13].

CMT4C4 disease associated with the S194X nonsense mutation begins in early childhood (mean 3.2±1.4 years). Some of those patients were born as floppy infants. The majority of patients presented with chest and spinal deformity.

The profound proximal muscle weakness observed in the CMT4C4 (S194X) disease resulted in a loss of ambulance even in early childhood. At the electrophysiological level, the patients display greatly reduced CMAPs in the upper limbs with no response at all in the lower limbs [5, 7, 9, 16].

A second recurrent *GDAP1* gene mutation, i.e., Q163X has been shown to be the most frequent *GDAP1* mutation in North American Hispanic families, again suggesting the possibility of a founder effect [8].

In a subsequent study, the Q163X mutation was identified as an ancient *GDAP1* variant with an estimated origin dating back to 33,000 years ago. A study encompassing 13 chromosomes from Spaniards and six Hispanic North Americans provided evidence for a common haplotype [12].

Patients harboring the Q163X mutation also manifest with a severe CMT4C4 phenotype. Characteristically, the majority of them display hoarseness [18].

At the electrophysiological level, the CMT4C4 disease caused by the Q163X mutation resembles the CMT phenotype associated with the Ser194X variant.

The patients harboring the Q163X/S194X compound mutation present with an extremely severe phenotype, leading to a loss of ambulance even by the age of 12 years [18].

In contrast to the CMT4C4 phenotype associated with S194X and Q163X mutations, the CMT4C4 entity resulting from the L239F mutation seems to represent a rather moderate phenotype.

Regarding age of onset, patients harboring the L239F mutation began to be symptomatic between 2 and 10 years. In particular, patients harboring the L239F mutation in the compound heterozygous state with M116T and N227D started to be affected from 10 years of age on. Recently, we have shown the M116T homozygous mutation to be associated with a moderate CMT phenotype [14].

It thus seems that some "milder" *GDAP1* variants may be associated with a later onset of CMT4A disease.

Obviously, patients harboring the L239F variant present with a milder phenotype than patients reported to date with *GDAP1* nonsense mutations.

The genotype of the patients harboring the L239F mutation in this study is not homogeneous (rather, three patients are homozygotes and seven compound heterozygotes). Thus, we cannot ignore the influence of other missense mutation on the clinical picture and natural history of CMT4C4 disease caused by the L239F mutation.

In fact, a coexistence of L239F and a nonsense E114fs mutation in the *GDAP1* gene has been shown to be associated with a severe CMT4C4 phenotype [2].

In our series of patients, the L239F mutation coexisted with a second missense *GDAP1* sequence variant in seven patients (R273G, R282C, N227D and M116T). Two of these mutations (R282C and M116T) occurring in the homozygous state have previously been reported as associated with a moderate CMT4C4 phenotype (available at www.molgen.ua.ac.be/CMTMutations), whereas the N227D and R273G mutations have not been reported to date.

Due to the presence of a second missense mutation in a compound heterozygote state with L239F the CMT4C4 phenotype observed in our series of patients may be mildly different from phenotypes associated with homozygous patients for the L239F mutation.

The exact mechanism of the L239F pathogenic effect remains to be determined. The L239F variant is located inside the C-terminal glutathione *S*-transferase domain of GDAP1, which was predicted in computer motif scan analysis [15].

The in silico analysis suggests impaired GDAP1 enzymatic activity, has been not confirmed via further biochemical studies [19].

The patients harboring 186fsX205 presented additionally with vocal cord paresis, unilateral elevation of the hemidiaphragm and decreased inspiration pressure [20]. Similarly, phrenic nerve and vocal cord palsy was detected in the CMT4C4 patients harboring Q163X, Q163X/T288fsX290 and Q163X/S194X [18].

In the study by Barankova et al. describing three CMT families carrying the L239F mutation, none of the patients aged 11–43 became wheelchair-bound [4].

Two brothers, A (III:12) and A(III:20), reported in this study lost ambulance at age 24, and 18.

We did not observe any CMT symptoms in the heterozygous carriers of the L239F mutation in the six families reported in this study. Similarly, in the previous studies reporting L239F mutations, no CMT symptoms were observed in the heterozygous carriers of this variant [2, 4]. This clearly indicates that the L239F mutation is transmitted in a pure recessive trait. This statement would seem to be important in the context of recently identified CMT2K patients harboring *GDAP1* gene mutations inherited in an autosomal dominant trait. It is noteworthy that the CMT2K affected patients manifest with a mild phenotype of sensory motor axonal neuropathy [10–12]. There is a question concerning the proportion of the CMT2K group accounts for in the cohort of patients harboring *GDAP1* gene mutations. In fact, up to the time when a first dominantly inherited *GDAP1* gene mutation was detected, the screening for *GDAP1* gene mutations was limited exclusively to the CMT families with a recessive trait of inheritance.

As with the previously reported Q163X and S194X variants for which a founder effect has been proved, so also in this study, we provided evidence for a common ancestor in the case of L239F mutation. Interestingly, the L239F mutation was never detected in patients of Hispanic origin (Q163X), or CMT patients from Maghreb countries (S194X).

While there is a possibility that, due to a founder effect, the occurrence of the L239F mutation is limited to the European population a lack of complete data prevents us from determining the precise geographical distribution of the L239F variant.

However, so far the L239F mutation seems to be the most frequent *GDAP1* pathogenic mutation in the European population.

Little is known of the frequency of the rare recessive mutations across Europe.

The peculiar geographical distribution of the L239F mutation in Europe provokes a question about the ancient groups which invaded the eastern part of Europe.

In this study, we estimated the age of the L239F mutation to be 22,000 years. Similarly, the Q163X mutation occurring in the Spaniards and Hispanic North Americans was dated to be 33,000 years old [12].

In conclusion, the combined findings in several families harboring the L239F mutation originating from a common ancestor reveal an emerging pattern that can be compared with other mutations. The L239F allele behaves like a pure

recessive allele and is associated with a milder phenotype than the other founder mutations Q163X and S194X on which sufficient data are available to make reliable genotype–phenotype correlation. Of course, other modifying factors will also play a role—as is clearly illustrated by the sibs harboring the same S194X/R310Q mutation, in which the proband was wheelchair-bound at age 15 year, whereas his sister was still ambulatory at age 26 years [3]. Only additional studies on a larger number of patients will allow the nature of these modifying factors to be addressed.

Acknowledgments The authors thank Ms. Jadwiga Kędzierska for her skillfull technical assistance. This study was supported by the Grant No. 402276336 from the Polish Ministry of Science and Higher Education to A.K. and by the Grant No. VUL-317 from the Bulgarian Ministry of Education and Science to V.G., I.T., and A.J. The work of the Belgian team was supported by the Fund for Scientific Research (FWO-Flanders), the Medical Foundation Queen Elisabeth (GSKE), the Association Belge contre les Maladies Neuromusculaires (ABMM), the Methusalem excellence grant of the Flemish Government, and the Interuniversity Attraction Poles P6/43 program of the Belgian Federal Science Policy Office (BELSPO). MZ and JB are supported by PhD fellowships of the FWO-Flanders.

References

1. Aarskog NK, Vedeler CA (2000) Real-time quantitative polymerase chain reaction. A new method that detects both the peripheral myelin protein 22 duplication in Charcot–Marie–Tooth type 1A disease and the peripheral myelin protein 22 deletion in hereditary neuropathy with liability to pressure palsies. Hum Genet 107:494–498
2. Ammar N, Nelis E, Merlini L, Barisic N, Amouri R, Ceuterick C et al (2003) Identification of novel GDAP1 mutations causing autosomal recessive Charcot–Marie–Tooth disease. Neuromuscul Disord 13:720–728
3. Azzedine H, Ruberg M, Ente D, Gilardeau C, Perie S, Wechsler B et al (2003) Variability of disease progression in a family with autosomal recessive CMT associated with a S194X and new R310Q mutation in the GDAP1 gene. Neuromuscul Disord 13:341–346
4. Barankova L, Vyhnalkova E, Zuchner S, Mazanec R, Sakmaryova I, Vondracek P et al (2007) GDAP1 mutations in Czech families with early-onset CMT. Neuromuscul Disord 17:482–489
5. Baxter RV, Ben Othmane K, Rochelle JM, Stajich JE, Hulette C, Dew-Knight S et al (2002) Ganglioside-induced differentiation-associated protein-1 is mutant in Charcot–Marie–Tooth disease type 4A/8q21. Nat Genet 30:21–22
6. Ben Othmane K, Hentati F, Lennon F, Ben Hamida C, Blel S, Roses AD et al (1993) Linkage of a locus (CMT4A) for autosomal recessive Charcot–Marie–Tooth disease to chromosome 8q. Hum Mol Genet 2:1625–1628
7. Birouk N, Azzedine H, Dubourg O, Muriel MP, Benomar A, Hamadouche T et al (2003) Phenotypical features of a Moroccan family with autosomal recessive Charcot–Marie–Tooth disease associated with the S194X mutation in the GDAP1 gene. Arch Neurol 60:598–604
8. Boerkoel CF, Takashima H, Nakagawa M, Izumo S, Armstrong D, Butler I et al (2003) CMT4A: identification of a Hispanic GDAP1 founder mutation. Ann Neurol 53:400–405
9. Bouhouche A, Birouk N, Azzedine H, Benomar A, Durosier G, Ente D et al (2007) Autosomal recessive axonal Charcot–Marie–Tooth disease (ARCMT2): phenotype–genotype correlations in 13 Moroccan families. Brain 130:1062–1107
10. Cassereau J, Chevrollier A, Gueguen N, Malinge MC, Letournel F, Nicolas G et al (2009) Mitochondrial complex I deficiency in GDAP1-related autosomal dominant Charcot–Marie–Tooth disease (CMT2K). Neurogenetics 10(2):145–150
11. Chung KW, Kim SM, Sunwoo IN, Cho SY, Hwang SJ, Kim J et al (2008) A novel GDAP1 Q218E mutation in autosomal dominant Charcot–Marie–Tooth disease. J Hum Genet 53:360–364
12. Claramunt R, Pedrola L, Sevilla T, Lopez DM, Berciano J, Cuesta A et al (2005) Genetics of Charcot–Marie–Tooth disease type 4A: mutations, inheritance, phenotypic variability, and founder effect. J Med Genet 42:358–365
13. Cuesta A, Pedrola L, Sevilla T, Garcia-Planells J, Chumillas MJ, Mayordomo F et al (2002) The gene encoding ganglioside-induced differentiation-associated protein 1 is mutated in axonal Charcot–Marie–Tooth type 4A disease. Nat Genet 30:22–25
14. Kabzinska D, Kochanski A, Drac H, Rowinska-Marcinska K, Ryniewicz B, Pedrola L et al (2006) A novel Met116Thr mutation in the GDAP1 gene in a Polish family with the axonal recessive Charcot–Marie–Tooth type 4 disease. J Neurol Sci 241:7–11
15. Marco A, Cuesta A, Pedrola L, Palau F, Marin I (2004) Evolutionary and structural analyses of GDAP1, involved in Charcot–Marie–Tooth disease, characterize a novel class of glutathione transferase-related genes. Mol Biol Evol 21:176–187
16. Nelis E, Erdem S, Van Den Bergh PY, Belpaire-Dethiou MC, Ceuterick C, Van GV et al (2002) Mutations in GDAP1: autosomal recessive CMT with demyelination and axonopathy. Neurology 59:1865–1872
17. Senderek J, Bergmann C, Ramaekers VT, Nelis E, Bernert G, Makowski A et al (2003) Mutations in the ganglioside-induced differentiation-associated protein-1 (GDAP1) gene in intermediate type autosomal recessive Charcot–Marie–Tooth neuropathy. Brain 126:642–649
18. Sevilla T, Cuesta A, Chumillas MJ, Mayordomo F, Pedrola L, Palau F et al (2003) Clinical, electrophysiological and morphological findings of Charcot–Marie–Tooth neuropathy with vocal cord palsy and mutations in the GDAP1 gene. Brain 126:2023–2033
19. Shield AJ, Murray TP, Board PG (2006) Functional characterisation of ganglioside-induced differentiation-associated protein 1 as a glutathione transferase. Biochem Biophys Res Commun 347:859–866
20. Stojkovic T, Latour P, Viet G, de Seze J, Hurtevent JF, Vandenberghe A et al (2004) Vocal cord and diaphragm paralysis, as clinical features of a French family with autosomal recessive Charcot–Marie–Tooth disease, associated with a new mutation in the GDAP1 gene. Neuromuscul Disord 14:261–264