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GDAP1 mutations in Czech families with early-onset CMT

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Abstract

Mutations in the *ganglioside-induced differentiation associated protein-1* gene (*GDAP1*) cause autosomal recessive (AR) demyelinating or axonal Charcot-Marie-Tooth neuropathy (CMT). In order to establish the spectrum and frequency of *GDAP1* mutations in Czech population, we sequenced *GDAP1* in 74 Czech patients from 69 unrelated families with early-onset demyelinating or axonal CMT compatible with AR inheritance. We identified three isolated patients with *GDAP1* mutations in both alleles. In one additional sporadic and one familial case, the second pathogenic mutation remained unknown. Overall, we detected two different mutations, a novel R191X nonsense and a L239F missense mutation. L239F previously described in a German–Italian family is a prevalent mutation in Czech population and we give evidence for its common ancestral origin. All Czech *GDAP1* patients developed involvement of all four limbs evident by the end of second decade, except for one isolated patient showing very slow disease progression. All patients displayed axonal type of neuropathy.

Keywords: HMSN type II; Charcot-Marie-Tooth disease; Autosomal recessive CMT2; *GDAP1*; R191X; L239F

1. Introduction

Charcot-Marie-Tooth disease (CMT) is a hereditary disorder of the peripheral nervous system characterized by progressive distal limb weakness, muscle atrophy and sensory loss, maximal in the lower extremities. Based on electrophysiological and histopathological findings, two CMT types are distinguished. The demyelinating type or CMT1 is characterized by the median nerve motor conduction velocity reduced below 38 m/s, demyelination and onion bulb formation in nerve biopsies. In the axonal type CMT2 nerve conduction velocities are normal or slightly reduced and nerve biopsies show axonal loss [1,2]. CMT1 and CMT2 are further sub-classified according to inheritance pattern (autosomal dominant (AD), autosomal recessive (AR), or X-linked) and the underlying molecular genetic cause [3].

Mutations in the *ganglioside-induced differentiation associated protein-1* (*GDAP1*) gene were originally reported in four AR Tunisian families with demyelinating neuropathy (CMT4A) and simultaneously by a different research group in three Spanish families diagnosed with AR axonal neuropathy and vocal cord paresis [4,5]. So far more than 20 different *GDAP1* mutations have been described (http://www.molgen.ua.ac.be/CMTMutations/) to cause severe early-onset AR neuropathy [6–13].

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The protein encoded by *GDAP1* shows similarity to glutathione-*S*-transferases (GST), enzymes involved in cellular antioxidative pathways [4,5,14]. Additionally, its localisation in neuronal mitochondrial membranes has been demonstrated and a role of mitochondrial dysfunction in the disease pathogenesis has been suggested [15].

We report the frequency, spectrum and phenotypic expression of *GDAP1* mutations in Czech CMT patients. We further give evidence for common ancestral origin of each mutation.

2. Patients and methods

2.1. Patients

Seventy-four patients from 69 unrelated Czech CMT families were screened for mutations in *GDAP1*. All selected families had pedigrees compatible with AR inheritance, i.e. the patients were either affected siblings born to healthy parents based on family history (nine families) or more often isolated cases. Consanguinity was not reported for any of the families. All the patients presented with the first neuropathy symptoms in the first decade. Both axonal (32 families) and demyelinating (37 families) CMT types were included. All patients were previously tested negative for CMT1A and 42 families (61%) also for mutations in MPZ and/or Cx32 genes (37 families (54%) were examined for MPZ and 24 (35%) for Cx32 gene).

2.2. Molecular studies

All six coding exons and intron–exon boundaries of *GDAP1* were PCR amplified using primers as described previously [4,5]. Purified PCR products (Exo I, SAP, Fermentas, Lithuania) were directly sequenced using four dye terminator chemistry (BigDye Terminator v3.1, ABI, USA) and analysed on an ABI 3100 capillary sequencer. Available relatives of the patients with sequence variations in the gene were examined genetically and clinically to confirm the segregation pattern. One hundred and fifty unrelated normal control subjects (300 chromosomes) were screened for the c.571C>T (R191X) mutation by primer extension analysis (SNaPshot, ABI, USA).

In the families carrying *GDAP1* mutations, haplotype analysis of the *GDAP1* locus was performed using two intragenic single nucleotide polymorphisms (SNPs), rs16938893 (c.507T>G, p.Ser169Ser) and rs3739345 and six microsatellite markers, D8S279, D8S286, D8S551, D8S1474, D8S548 and D8S1829, flanking the gene locus. The regions comprising the SNPs were directly sequenced. The microsatellite markers were PCR amplified using fluorescent labelled primers (http://www.gdb.org/) and the fragments were resolved on an ABI 310 sequencer. Allele frequencies were established by testing 50 Czech unrelated individuals carrying neither of the detected mutations (100 chromosomes). Probabilities of finding the L239F or R191X associated haplotypes among normal Czech chromosomes were calculated by multiplying the respective marker allele frequencies. Haplotypes of a previously reported German–Italian family [7] were compared with Czech patients carrying the same *GDAP1* mutation (c.715C>T, L239F).

For reverse transcription (RT) PCR experiments, RNA was extracted from peripheral blood, reverse transcribed (Promega), and PCR amplified with primer pairs spanning all coding exons. PCR products were visualized on 1.5% agarose gel and directly sequenced.

2.3. Clinical studies

Clinical and electrophysiological evaluations of the patients with *GDAP1* mutations and parent mutation carriers were performed according to standard procedures.

All tested individuals signed an informed consent and the study was approved by the Central Ethical Committee of the University Hospital Motol Prague.

3. Results

3.1. Mutation analysis

Two different point mutations, a novel c.571C>T (R191X) and a previously reported c.715C>T (L239F) [7], were detected in the coding region of *GDAP1*. At least one pathogenic mutation was found in six patients from five apparently unrelated families representing 7.2% out of the 69 families tested. As there was no *GDAP1* mutation detected among demyelinating cases, the frequency in CMT2 families is estimated to be 15.6%. Mutations in both alleles were detected in three isolated CMT patients (4.3% and 9.4% of CMT2 families), in one compound heterozygote for c.715C>T and c.571C>T, and in two homozygotes for c.715C>T. Heterozygous state of c.715C>T in the parental generation of the two latter families could be confirmed only in one parent that was available for the examination. The second pathogenic mutation remained unidentified in one isolated patient heterozygous for c.715C>T from family 4 and in two siblings from family 5, both heterozygous for c.571C>T. In both families the mutant allele was inherited from an unaffected parent as illustrated by clinical and electrophysiological data given in Tables 3 and 4. The c.715C > T (L239F) detected in six out of eight mutant alleles is a prevalent mutation in the Czech population. The mutation has been already described in a compound heterozygous state in one German-Italian family [7].

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In order to identify the second causative mutation in family 5, RT-PCR analysis was performed. No exon deletions and no abnormal splicing were detected.

The novel mutation c.571C>T (R191X) was not detected in 150 control individuals.

3.2. Haplotype analysis

To find out whether each of the detected *GDAP1* mutations originates from a common ancestor, we reconstructed haplotypes for the German–Italian and the five Czech *GDAP1* families by analysis of six microsatellite markers spanning the gene locus and two intragenic SNPs. Fig. 1 summarizes the haplotypes observed. Haplotype phase could not be determined completely in the patient from family 4.

Detailed family history assessment of the German L239F carrier revealed her parents' origin from Western Pommerania, a region near the German–Polish border. Five Czech L239F chromosomes originate from the Moravia region in the eastern part of the Czech Republic and one from the western part of the country. Four Czech chromosomes (A, B, C and E from families 1, 2 and 3) and the German chromosome carrying the L239F variant showed an identical core haplotype between D8S286 through D8S548 (~560 kb). The probability of finding the haplotype among normal chromosomes is very low, i.e. less than 7.34×10^{-6}. Accidental coincidence of the marker alleles in the L239F chromosomes is therefore unlikely. Czech chromosome A and the German chromosome shared the complete haplotype including all analysed markers (~2970 kb).Compared to the core haplotype, chromosome D revealed an allele one repeat shorter at marker D8S1474 but shared the rare allele 278 at marker D8S551 (not found in 100 chromosomes). Chromosome F coincided only at marker D8S286 and at both intragenic SNPs assuming its haplotype to be T–G.

All the markers in families 3 and 5 segregated with R191X. The probability of occurrence of the R191X associated haplotype in the Czech population is even lower, i.e. less than 6.59×10^{-8}.

3.3. Clinical and electrophysiological findings in GDAP1 patients

3.3.1. Family 1

In the patient from family 1, early motor development was normal. At the age of 4 years he developed bilateral pes planovalgus. Motor nerve conduction studies performed at the peroneal and tibial nerves at age 5 showed severely decreased or absent compound muscle action potentials (CMAPs) and normal nerve conduction velocities (NCVs). At ages 6 and 7, the patient underwent surgical corrections of the foot deformity. Neurological

L239F

Family/ Chromosome	Origin	D8S279	*GDAP1* D8S286	D8S551	rs3739345	c.507T/G	D8S1474	D8S548	D8S1829
1/A	Moravia	250	229	278	T	G	177	234	113
1/B	Moravia?[a]	246	229	278	T	G	177	234	113
2/C	Moravia[b]	246	229	278	T	G	177	234	103/111
2/D	west.Bohemia	226	229	278	T	G	181	234	103/111
3/E	Moravia	226	229	278	T	G	177	234	111
4/F	Moravia	250/238	229	276	T/A	G/T	185	232	113
Marker allele freq. in controls			0.219	**<0.01**	0.343	0.370	0.075	0.352	
German-Italian	West.Pommerania	250	229	278	T	G	177	234	113

R191X

Family	Origin	D8S279	*GDAP1* D8S286	D8S551	rs3739345	c.507T/G	D8S1474	D8S548	D8S1829
3	midd.Bohemia	226	235	266	T	G	177	248	113
5	midd.Bohemia	226	235	266	T	G	177	248	113
Marker allele freq. in controls		0.323	0.042	0.229	0.343	0.370	0.075	**<0.01**	0.223

Fig. 1. Disease haplotypes in five Czech and the German–Italian family with *GDAP1* mutations. [a]DNA of the patient's father was not available for examination, paternity was not proven. [b]DNA of the patient's mother was not available for examination.

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examination at age 8 revealed marked distal lower limb (LL) weakness and atrophies, absent Achilles tendon reflexes and decreased patellar reflexes, mild wasting of the hand muscles with slightly reduced strength and diminished upper limb (UL) reflexes.

3.3.2. Family 2

The patient from family 2 started to walk at 1 year of age. At age 4, walking at the outer edge of the left foot was noted followed by bilateral development of pes cavovarus. The deformity was treated by orthopaedic surgery at age 7. On examination at age 12, the patient showed distal LL weakness and marked atrophies of the foot muscles and calves, gait with foot drop, finger tremor and spine deformity (scoliosis and chest hyperkyphosis). At age 19, LL atrophies reached distal parts of the thighs, Achilles tendon reflexes were absent and patellar reflexes diminished. There was mild atrophy of the right thenar and normal UL reflexes. At age 29, atrophies of the interosseal muscles were noted without loss of strength. From age 35, the patient states weakening of the hands and using a cane for longer distances because of marked proximal LL muscle weakening.

3.3.3. Family 3

Early motor milestones of the patient from family 3 were normally acquired by 1 year of age. At the age of 2 years, his parents noted tip toe walking. At age 4, bilateral prolongation of the Achilles tendons was performed. In the first decade, the gait deteriorated by progressive distal LL weakness and pes equinovarus deformity and was only possible with the aid of crutches. The ambulation much improved after the second orthopaedic surgery at age 13 and support was further required only for longer distances. Weakness and wasting of the small hand muscles and clawing of the fingers were already apparent before the start of school attendance at age 8.

3.3.4. Family 4

In the patient from family 4, the first CMT symptoms were observed at the age of 4 years, when he presented with cavus foot deformity. At ages 12 and 13, he underwent its surgical corrections. The first neurological and electrophysiological examination at age 39 showed well preserved foot dorsiflexion, preserved patellar reflexes, normal clinical and electrophysiological findings at the UL and diminished CMAPs and sensory nerve action potentials (SNAPs) at the LL. The patient has no major complaints except of ankle instability and frequent ankle distortions.

3.3.5. Family 5

The older affected sibling from family 5 (5/1) exhibited bilateral Achilles tendon shortening, high arched foot and clumsy gait at the age of 6 years. Examination at age 15 showed distal LL atrophies, complete LL areflexia, diminished UL reflexes, hand tremor and scoliosis. The Achilles tendons were surgically lengthened at age 16. At age 19, atrophies of the small hand muscles were noted, but strength was well preserved. Examination at age 22 revealed preserved foot and toe dorsiflexion, UL muscle atrophies up to the distal forearms and only mild hand muscle weakening with preserved dexterity. His sister (5/2) showed normal motor milestones. At age 6, tip toe gait and inward twisting of the right foot became apparent. She developed early pes equinovarus right, pes planovalgus left and steppage gait. Neurological examination at age 13 showed hand muscle atrophies and weakening, diminished UL reflexes and scoliosis. The right foot deformity was surgically treated at age 16. Examination at age 16 revealed severe LL muscle wasting up to the lower thighs, gait with marked foot drop, using Gower's manoeuvre when rising from the floor, marked hand muscle atrophies, weakening and clumsiness with beginning finger retraction and complete areflexia.

Further clinical and electrophysiological data are summarized in Tables 1 and 2.

4. Discussion

We identified mutations in at least one *GDAP1* allele in 7.2% of unrelated Czech CMT families with early-onset axonal or demyelinating neuropathy and disease transmission compatible with autosomal recessive inheritance pattern. Pathogenic mutations in both alleles were detected in 4.3% of the families. A detection rate of 5% in isolated cases makes testing sporadic CMT patients for *GDAP1* mutations reasonable in the Czech population [7,16]. Direct sequencing of all coding exons and adjacent intronic regions of the gene did not reveal a second pathogenic mutation in a significant portion of the *GDAP1* families (2 out of 5, 40%).

We detected two distinct *GDAP1* mutations, a novel nonsense (c.571C>T, R191X) and a previously described missense (c.715C>T, L239F) mutation [7]. R191X predicts a truncation of the GDAP1 protein in front of the functional GST_C domain and the transmembrane domains, which are necessary for the proper localization of GDAP1 to mitochondrial membranes [14,15]. Thus, we speculate on a loss of function effect of R191X.

All our *GDAP1* patients showed normal motor milestones before the first year of age followed by early development of severe foot deformity between 2 and 6 years that required surgical treatment. Consistent with previous reports, most of our patients developed severe motor deficit in the distal lower limbs within the first decade. However, none of the patients aged 11–43 became wheelchair-bound as frequently reported in *GDAP1* patients [6–9,11,13,16,17]. One patient has been using a cane due to proximal LL weakness only since the fourth decade. Distal upper limb muscle involvement of varying

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Table 1
Clinical summary in six Czech patients with *GDAP1* mutations

Family/patient	1	2	3	4	5/1	5/2
Genotype	L239F+L239F	L239F+L239F	L239F+R191X	L239F+?	R191X+?	R191X+?
Age (yr)/gender	11/M	43/M	17/M	41/M	22/M	18/F
Foot deformity	Pes planovalgus	Pes cavovarus	Pes planovalgus	Pes cavus	Pes cavus	Pes equinovarus right, pes planovalgus left
Foot dorsiflexion right, left (MRC)	2,3/5	0,0/5	0,0/5	4+,4+/5	4,4/5	3,1/5
Proximal LL weakness right, left (MRC)	Knee flexion 4,4-/5	Knee flexion 3,2/5, knee extension 4+,4/5, hip extension 4,4/5	No	No	No	Hip and knee flexion and extension 4-,4-/5
LL atrophy	Severe, up to distal thighs	Severe, up to lower halves of thighs	Severe, up to distal thirds of thighs	Moderate, up to distal thighs	Severe, up to distal parts of thighs	Severe, including lower parts of thighs
LL reflexes	Absent	Absent	Absent	Achilles tendon reflexes absent	Absent	Absent
Walking ability	No support	Cane	Crutches	No support	No support	No support
UL atrophy	Mild at hands	Moderate at hands, mild at forearms	Severe at hands-claw-hand, moderate at distal halves of forearms	No	Mild at hands and forearms	Moderate at hands and forearms
Distal UL weakness right, left (MRC)	Hand grip 4,4/5, wrist extensors 4+,4+/5, wrist flexors 4-,4+/5	Hand grip 4+,4+/5	Hand grip 3,3/5, wrist extensors 4-,3/5, wrist flexors 4+,4/5, loss of dexterity	No	Hand grip 4+,4+/5	Severe hand grip weakening, exact data NA
Proximal UL weakness (MRC)	No	No	No	No	No	No
UL reflexes	Normal	Decreased	Decreased	Normal	Decreased	Decreased
Sensory impairment	Mild loss of touch sense at LL	No	Moderate loss of touch sense and mildly decreased joint position sense at LL	No	Mild loss of touch sense at LL, moderate loss of vibration sense at LL	No loss of touch sense, further data NA
Other	No	Scoliosis	Tongue tremor	Scoliosis	Scoliosis	Scoliosis

M, male; F, female; MRC, Medical Research Council; LL, lower limbs; UL, upper limbs; NA, not available.

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Table 2
Summary of electrophysiological findings in six Czech patients with *GDAP1* mutations

Family/patient		**1**	**2**	**3**	**4**	**5/1**	**5/2**	**normal**
Age (yr)		8	43	15	41	23	18	
Motor nerves								
Median nerve	DML (ms)	3.9	3.8	**NR**	3.6	**4.9**	NA	<4.0
	MNCV (m/s)	64	59		53	**45**		>51
	CMAP (mV)	6.4	8.8		8.2	7.1		>4.2
Ulnar nerve	DML (ms)	**3.4**	**3.4**	**4.1**	2.3	**3.2**	**3.6**	<2.8
	MNCV (m/s)	67	56	**40**	60	51	**49**	>50
	CMAP (mV)	**4.6**	**3.6**	**0.9**	7.1	5.8	**1.9**	>5.5
Peroneal nerve	DML (ms)	**NR**	**NR**	**NR**	3.2	NA	NA	<5.0
	MNCV (m/s)				45			>39
	CMAP (mV)				3.0			>2.0
Tibial nerve	DML (ms)	5.2	**NR**	**NR**	5.3	**NR**	**NR**	<5.5
	MNCV (m/s)	44			**38**			>40
	CMAP (mV)	**0.5**			**0.5**			>2.5
Sensory nerves								
Median nerve	SNCV (m/s)	**NR**	**44**	**NR**	57	**NR**	**NR**	>48
	SNAP (μV)		15		14			>10
Ulnar nerve	SNCV (m/s)	**NR**	**48**	**NR**	55	NA	NA	>48
	SNAP (μV)		15		**10**			>10
Sural nerve	SNCV (m/s)	**NR**	40	**NR**	**38**	**NR**	**NR**	>38
	SNAP (μV)		**2.0**		5.0			>4.4

DML, distal motor latency; CMAP, compound muscle action potential; MNCV, motor nerve conduction velocity; SNAP, sensory nerve action potential; SNCV, sensory nerve conduction velocity; NR, not recordable; NA, not available.
Pathological values are in bold.

Table 3
Clinical summary in parent carriers of L239F and R191X in two heterozygous families

Family	**4**	**5**
Genotype	L239F	R191X
Age (yr)/Gender	72/M	57/M
Foot deformity	No	No
Foot dorsiflexion right, left (MRC)	5,5/5	5,5/5
Proximal LL weakness right, left (MRC)	No	No
LL atrophy	No	No
LL reflexes	Decreased	Normal
Walking ability	No support	No support
UL atrophy	No	No
Distal UL weakness right, left (MRC)	No	No
Proximal UL weakness (MRC)	No	No
UL reflexes	Normal	Normal
Sensory impairment	Mild loss of vibration sense at LL	Mild loss of touch sense in median nerve distribution

M, male; MRC, Medical Research Council; LL, lower limbs; UL, upper limbs.

extent became evident by the end of the second decade and appeared to be severe only in two patients [8–11,17]. Moreover, we observed very slow disease progression in the 41 year old heterozygous carrier of the L239F missense mutation from family 4 showing only mild weakening of the toe and foot dorsiflexion. The mutation was reported in a compound heterozygote state with Glu114fs by Ammar et al. in a patient who seemed to be more severely affected compared to our L239F patients due to loss of ambulation at age 11 [7]. We also noted strikingly different disease progression and severity in the two siblings from family 5, heterozygotes for the R191X nonsense mutation. All the *GDAP1* patients displayed an axonal type of neuropathy. None of them had vocal cord paresis, previously reported to be associated with CMT2 and *GDAP1* mutations [11,12,17]. No patient showed respiratory insufficiency due to diaphragm paralysis [11,12]. Regarding the better walking ability in our patients, we admit positive impact of the early orthopaedic surgery performed.

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Table 4
Summary of electrophysiological findings in parent carriers of L239F and R191X in two heterozygous families

Family		**4**	**5**	**normal**
Genotype		L239F	R191X	
Age (yr)		72	57	
Motor nerves				
Median nerve	DML (ms)	3.6	**5.6**	<4.0
	MNCV (m/s)	54.1	**50.5**	>51
	CMAP (mV)	10.3	5.5	>4.2
Ulnar nerve	DML (ms)	2.6	**2.8**	<2.8
	MNCV (m/s)	56.2	57.1	>50
	CMAP (mV)	10.2	9.9	>5.5
Peroneal nerve	DML (ms)	4.6	4.7	<5.0
	MNCV (m/s)	46.7	48.3	>39
	CMAP (mV)	5.1	4.9	>2.0
Tibial nerve	DML (ms)	3.9	NA	<5.5
	MNCV (m/s)	46.2		>40
	CMAP (mV)	11.8		>2.5
Sensory nerves				
Median nerve	SNCV (m/s)	51.8	**36.4**	>48
	SNAP (μV)	14.2	**5.9**	>10
Ulnar nerve	SNCV (m/s)	**47.1**	48.3	>48
	SNAP (μV)	16.7	**7.7**	>10
Sural nerve	SNCV (m/s)	46.6	40.6	>38
	SNAP (μV)	6.3	5.4	>4.4

DML, distal motor latency; CMAP, compound muscle action potential; MNCV, motor nerve conduction velocity; SNAP, sensory nerve action potential; SNCV, sensory nerve conduction velocity; NR, not recordable; NA, not available.
Abnormal values are in bold.

L239F accounted for 75% of the mutant alleles in the Czech population. Haplotype analysis of the German–Italian and four Czech families carrying the L239F mutation indicated a common ancestral founder. The ancestors of the German L239F carrier lived in Western Pommerania, near todays German–Polish border. This area was historically populated by Germans and Slavs with a high rate of intermarriage. Considering the lack of systematic screening data on spectrum and frequency of *GDAP1* mutations in Germany and Poland and historical and geographical proximity of all three countries, closer specification of the mutation origin is not possible. The less frequent nonsense mutation R191X was also found to be associated with a common haplotype, which included remote markers as well, indicating its later origin.

In the view of a recent Claramunt et al. report on autosomal dominant effect of some *GDAP1* mutations, we examined clinically and electrophysiologically parent mutation carriers from the two heterozygous families [16]. Our findings do not support dominant transfer of the disease and suggest that a second mutation in another region (i.e. in the promotor or deep intronic regions) of *GDAP1* or in another gene caused the CMT phenotype in the heterozygous patients. Haplotype analysis in these families showed notable difference in the ''nonmutant'' chromosome haplotypes (differing in five out of six microsatellite markers tested, data not shown). This might indicate the existence of two additional *GDAP1* mutations in the Czech population provided that we do not consider a digenic cause of the disease. In family 5, RNA analysis excluded large deletions and intronic mutations affecting splicing. After all, we cannot rule out co-occurence of a heterozygous *GDAP1* mutation and CMT with different genetic cause, e.g. in the L239F heterozygote from family 4 with mild clinical phenotype.

The results of this study will help to optimize the molecular-genetic examination algorithm in Czech CMT patients. Testing for *GDAP1* mutations should be performed in patients with axonal CMT in preference to demyelinating cases. Targeted mutation analysis to L239F followed by R191X using a cheaper specific mutation directed method appears to be effective. In case of negative or mono-allelic mutation findings, sequencing of the whole gene should be completed.

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