Journal of the Neurological Sciences 402 (2019) 156–161

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns

A very mild phenotype of Charcot-Marie-Tooth disease type 4H caused by two novel mutations in *FGD4*

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ARTICLE INFO

Keywords:
Charcot-Marie-Tooth
CMT
FGD4
CMT4H
Neuropathy

ABSTRACT

Background: Mutations in the *FGD4* gene cause an autosomal recessive demyelinating peripheral neuropathy referred to as CMT4H, characterized by its onset in infancy or early-childhood and its slow progression.
Methods: The clinical and genetic status of two patients with CMT4H was studied, performing genetic testing with a panel of genes and analysing *FGD4* mRNA expression by quantitative PCR.
Results: Two novel *FGD4* variants (c.514delG and c.2211dupA) were identified in two mildly affected Spanish siblings with CMT4H, and with disease onset in late adolescence/adulthood (one of them remaining asymptomatic at 20). On examination, foot deformity was observed without weakness or sensory involvement, and in the muscles of the lower extremities magnetic resonance imaging showed no fat replacement. Further analysis of *FGD4* expression in peripheral blood suggested that neither mutation affected splicing, nor did they affect the dosage of *FGD4* mRNA (compared to a healthy control). It was predicted that each allele would produce a truncated protein, p.Ala172Glnfs*28 (c.514delG) and p.Ala738Serfs*5 (c.2211dupA), the latter containing all the functional domains of the native protein.
Conclusions: The conservation of functional domains in the proteins produced from the *FGD4* gene of two patients with CMT4H, could explain both the milder phenotype and the later disease onset in these patients. These results expand the clinical and mutational spectrum of *FGD4*-related peripheral neuropathies.

1. Introduction

The classification of Hereditary Motor and Sensory Neuropathies (HMSN), commonly referred to as Charcot-Marie-Tooth (CMT) disease, as either demyelinating or axonal is usually based on electrophysiological or pathological features. Autosomal recessive

Abbreviations: HMSN, Hereditary motor and sensory neuropathies; CMT, Charcot-Marie-Tooth disease; *FGD4*, frabin gene; CMT4H, CMT type 4H; CMTNS-v2, CMT Neuropathy Score version 2; CMTPedS, CMT Paediatric Scale; MRI, Magnetic resonance imaging; dHMN, distal hereditary motor neuropathy; ALS, Amyotrophic lateral sclerosis; RT-PCR, reverse transcription-PCR; qPCR, quantitative PCR; NCV, Nerve conduction velocity; CMAP, Compound motor action potential; SNAP, Sensory nerve action potential; DL, Distal latency; NR, Non recordable; ns, No significant; FAB, F-actin binding; DH, Dbl homology; PH, Pleckstrin homology; FYVE, Fab 1, YOTB, V ac 1, and EEA1 zinc finger domain; NMD, Nonsense-mediated decay

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https://doi.org/10.1016/j.jns.2019.05.015
Received 22 March 2019; Received in revised form 4 May 2019; Accepted 14 May 2019
Available online 15 May 2019
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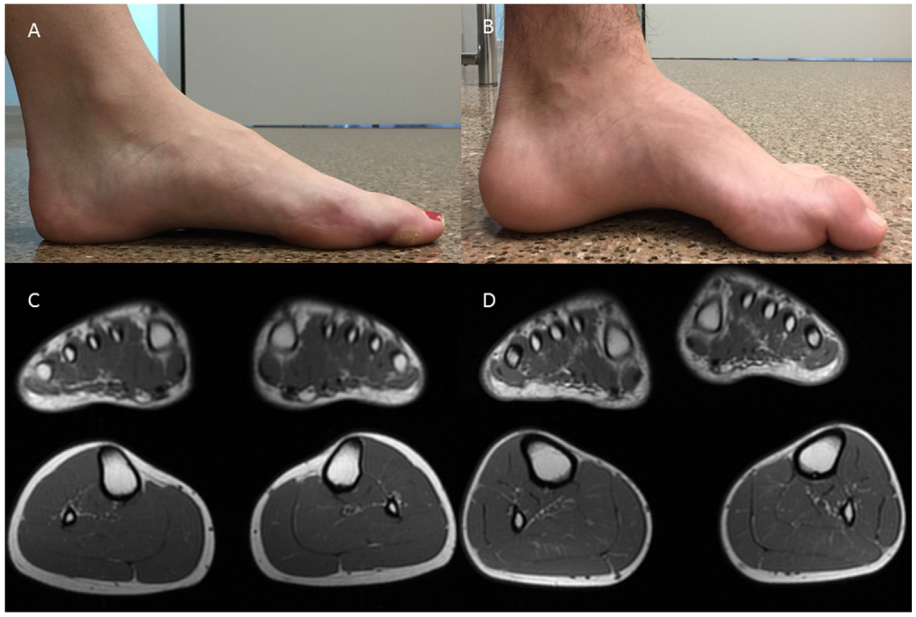

Fig. 1. Clinical images and muscle MRI (T1 weighted images shown). A Mild pes cavus of patient II:1. B Moderate pes cavus with hammer toes of patient II:2. C Muscle MRI of patient II:1 showing no fat replacement at the level of the feet and calf. D Muscle MRI of patient II:2 showing no significant abnormalities of either the feet or calf.

demyelinating forms of CMT (CMT4) tend to produce a more severe phenotype than autosomal dominant demyelinating forms (CMT1), and with an earlier onset [1,2]. In 2007, biallelic mutations in the frabin gene (*FGD4*) that encodes a Rho GTPase guanine nucleotide exchange factor were identified as the cause of CMT type 4H (CMT4H) [3]. Since then, 31 patients from 19 families with CMT4H have been reported, the vast majority of whom experienced symptoms during childhood (usually before the age of 3) or presented with delayed walking [4]. In some patients, scoliosis [3,10,12,13,15,16,18], and sensory ataxia [4,11,13] have been observed. Individual CMT4H cases have recently been reported with spinal syringomyelia [11], pupil asymmetry [8], multiple cranial nerve involvement [14], or cerebellar dysfunction [18]. Here we describe two patients with CMT4H, one of whom was asymptomatic at the age of 20, that carried two novel, compound heterozygous frameshift mutations in *FGD4*. An analysis of these mutations sheds light on their possible implications for the phenotype of these patients.

2. Material and methods

2.1. Subjects

The two affected individuals are siblings born to a healthy non-consanguineous Spanish couple (Fig. 2A) with no family history of neuromuscular disease. A thorough neurological examination was carried out at the Neuromuscular Unit of the Department of Neurology of the Hospital Universitari i Politècnic La Fe (HUPLF), where the two individuals were subsequently followed. The phenotype of each individual was studied using two scales designed to measure disability in inherited sensory-motor neuropathies: the CMT Neuropathy Score version 2 (CMTNS-v2) and the CMT Paediatric Scale (CMTPedS). CMTNS-v2 includes neurophysiology items and it has been validated for patients older than 16. The scores obtained range from 0 to 40 and patients are classified as mild with a CMTNS-v2 score ≤ 10 [5]. CMTPedS is an 11-item scale for patients between 3 and 20 years of age in which the scores range from 0 to 44, with a score of 0 representing unaffected patients [6]. Flexibility of ankle joint dorsiflexion was measured weight bearing using the lunge test. No Achilles retraction is present if lunge test $> 35^{\circ}$ [7]. Both patients also underwent comprehensive electrophysiological studies and muscle magnetic resonance imaging (MRI) was performed on the hips, thighs, calves and feet using a 3-T system (Siemens Vision, Siemens, Germany). After obtaining informed consent, blood samples were collected from all five members of the family and DNA was extracted using standard procedures. This study was approved by the institutional research board (IRB) at the Health Research Institute Hospital La Fe.

2.2. Molecular studies

Patient II:2 was tested for our customized panel of 119 genes using SureSelectQXT technology for Illumina (Agilent Technologies, Santa Clara, CA, USA), a panel that includes genes associated with CMT, distal hereditary motor neuropathy (dHMN), and familial amyotrophic lateral sclerosis (fALS). Cascade testing (using Sanger sequencing) was performed for other family members. To study the possible effect of these two variants on mRNA expression, we analysed the cDNA products generated from the *FGD4* mRNA extracted from the peripheral blood of patient II:2 and his progenitors. After extracting total RNA using the PAXgene Blood RNA kit (QIAGEN, Valencia, CA, USA), cDNA was obtained and amplified by reverse transcription-PCR (RT-PCR) using the qScript cDNA SuperMix (Quantabio, Beverly, MA, USA). The presence of mutations at the cDNA level was determined by Sanger sequencing with the following forward and reverse primers: 5' CAGATCTCATCAG TCGCTTTG and 5' TGCTTCTTCCAACAGTTTGC to study the c.514delG variant; and 5' CATAAGTGGATTCACAGACAGTG and 5' GAATGACTC TGCACACTAATTTC to study the c.2211dupA variant. To analyse the relative amounts of the cDNA products, quantitative PCR (qPCR) was performed using the PerfecTa SyberGreen Mix (Quantabio, Beverly, MA, USA) and the following forward and reverse *FGD4* primers: 5' TCAGATCTCATCAGTCGCTTTG and 5' ACAGCAGACTCTTTCTTCAAA TCA. A healthy control sample was used for calibration and *GAPDH* was used as the reference gene for normalization. An unpaired *t*-test was used to compare the cDNA doses in II:2 with those in the rest of the samples.

3. Results

3.1. Clinical picture

The older of the two patients (Fig. 2A, II:1) studied here is a 20-year-old woman who remains completely asymptomatic, displaying no difficulties in running, jumping or handling small objects. Since childhood, she had trained for 10 h each week as a rhythmic gymnast, with no limitations. At the age of 16 she began to perform highly demanding

Table 1
Motor and sensory nerve conduction studies.

CMAP	Patient II:1 (20 yo, F)			Patient II:2 (17 yo, M)		
Nerve	DL (ms)	Amplitude (mV)	NCV (m/s)	DL (ms)	Amplitude (mV)	NCV (m/s)
Median	5.5 [4.5]	11.6 [5.9]	21.1 [53]	6.45 [4.5]	4.3 [5.9]	16.5 [49]
Ulnar	4.6 [3.7]	6.9 [7.9]	22.2 [52]	5.0 [3.7]	5.3 [7.9]	14.9 [52]
Peroneal	9.2 [6.5]	3.1 [2.6]	15.0 [43]	12.2 [6.5]	3.8 [2.6]	12.0 [43]

SNAP	Patient II:1 (20 yo F)		Patient II:2 (17 yo M)	
Nerve	Amplitude (μV)	NCV (m/s)	Amplitude (μV)	NCV (m/s)
Median	15.9 [17]	27.8	8.2 [17]	22.7
Ulnar	6.4 [14]	27.9	2.4 [14]	22.3
Radial	18 [7]	27.8	5.2 [7]	22.4
Sural	3.2 [4]	24.0	NR [4]	NR

CMAP, Compound motor action potential; DL, Distal latency; ms, millisecond; mV, millivolts; NCV, Nerve conduction velocity; m/s, metres per second; SNAP, Sensory nerve action potential; μV, microvolts; NR Non recordable. Lower limits of onset-to-peak amplitudes and velocities are shown as mean – 2 SD in box brackets. Reference values were extracted from Chen S, Andary M, Buschbacher R, Del Toro D, Smith B, So Y, et al. Electrodiagnostic reference values for upper and lower limb nerve conduction studies in adult populations. Muscle Nerve 2016;54:371-7.

cardiovascular exercise three times weekly. She does not need to use any special apparatus or require special footwear, although areflexia and mild pes cavus were detected at 19 years of age (Fig. 1A), as well as mild retraction of the Achilles tendons (lunge test 30° on the left side and 25° on the right) that mildly affected heel walking. The patient's motor balance, sensory examination and muscle mass were normal, and neither pupillary abnormalities nor scoliosis were detected. The CMTNS-v2 score for this patient was 0, while the total CMTPedS score was 1 (scoring 1 on gait). Electrophysiological studies showed slow motor and sensory nerve conduction velocities (NCVs) in all nerves. Amplitude of compound muscle action potentials (CMAPs) and sensory nerve action potentials (SNAPs) was normal or marginally reduced, respectively (Table 1). The muscle MRI performed at age 19 revealed no fat replacement or volume changes, not even in her feet (Fig. 1C).

The index patient (Fig. 2A, II:2) was a 17-year-old male who walked unassisted at 12 months of age, yet he began to suffer from dorsal kyphoscoliosis and increased plantar arch when he was 11 years old. He was prescribed shoe-inserts when aged 12 and indicated he was symptomatically stable since the age of 13. From the age of 3 until he was 16, he had been playing football for up to 12 h per week, having suffered no injuries or experiencing difficulties in keeping up with his peers. When examined at the age of 16, areflexia, moderate pes cavus and hammer toes were noted (Fig. 1B), and a lunge test was compatible with mild Achilles tendon retraction (20° on both sides). No weakness, amyotrophy, pupillary size abnormality or sensory deficits were observed. He scored 4 in the CMTNS-v2, mainly because of the neurophysiological alterations, and his total CMTPedS Score was 3, scoring 2 on balance and 1 on gait. His nerve conduction was similar to that of his sister, although his motor NCVs were slower, and the amplitude of his CMAPs and SNAPs was smaller (Table 1). Muscle MRI of lower extremities at the age of 16 did not show any significant abnormalities (Fig. 1D).

3.2. Genetic analysis

Genetic testing of the proband (Fig. 2A: II:2) revealed two novel candidate variants in the *FGD4* gene (NM_139241.2): c.514delG and c.2211dupA. The other three variants were identified in heterozygous status: one missense variant in *PLEKHG5* (rs140202670), and a synonymous change in both *SBF1* and *UBQLN2* (rs180800708 and rs142250604, respectively). Their allele frequency were relatively high in the control database consulted (ExAC and gnomAD). Sequence variants in *SBF1* and *UBQLN2* were classified as benign based on the American College of Medical Genetics and Genomics criteria. The variant in *PLEKHG5* gene was ruled out as disease-causing because known mode of inheritance is autosomal recessive. In contrast, both *FGD4* mutations were absent in the control and mutation databases (ExAC, gnomAD, NCBI, ClinVAR and HGMD) and segregated with the disease within the family. Segregation analysis confirmed that these two changes exist in *trans* and while the unaffected mother harboured the c.514delG variant in heterozygosis, the healthy father carried the c.2211dupA variant in heterozygosis. Patient II:1 also carried both mutations, whereas the healthy sibling (Fig. 2A: II:3) did not carry either. The presence of both changes in heterozygosis was confirmed by analysing the *FGD4* mRNA isolated from patient II:2, sequencing two different cDNA fragments and thereby ruling out any alterations in the mRNA sequence adjacent to both mutations (Fig. 2B). In addition, qPCR analysis of *FGD4* mRNA in patient II:1 showed that there were no significant differences in the *FGD4* mRNA dosage in the tissue examined relative to the healthy control, the unaffected carriers or patient II:2 (Fig. 2C). It was predicted that the two variants identified each produce a truncated protein, the p.Ala172Glnfs*28 (c.514delG) that lacks functional domains and the p.Ala738Serfs*5 (c.2211dupA) that contains all of these (Fig. 3).

4. Discussion

We have identified two novel frameshift mutations in the *FGD4* gene of two siblings, both diagnosed with a demyelinating neuropathy with later onset and a milder phenotype than those reported previously. Analysis of mRNA expression did not show any effect on splicing or on *FGD4* dosage, which might reflect nonsense-mediated mRNA decay (NMD).

Since the first two families with CMT4H were described [3], this condition has been regarded as a very early onset demyelinating disease with a severe phenotype that involves delayed walking, scoliosis, and severe muscle weakness associated with an early loss of ambulation. However, as the number of CMT4H families has increased (now reaching 19 in total), milder forms of this condition have also been described. In addition, a few patients with associated sensory ataxia [4,11,13], spinal syringomyelia [11], pupil abnormality [8], cranial nerve involvement [14], or cerebellar dysfunction [18] have been reported, hence broadening the known CMT4H phenotype. A clinical feature common to all patients is onset during infancy and slow disease progression. As such, all patients began to experience symptoms before they reached 9 years of age [8–18]. However, our 20-year-old patient was still asymptomatic although the symptoms began to appear in her brother during his second decade. Muscle MRI findings support the

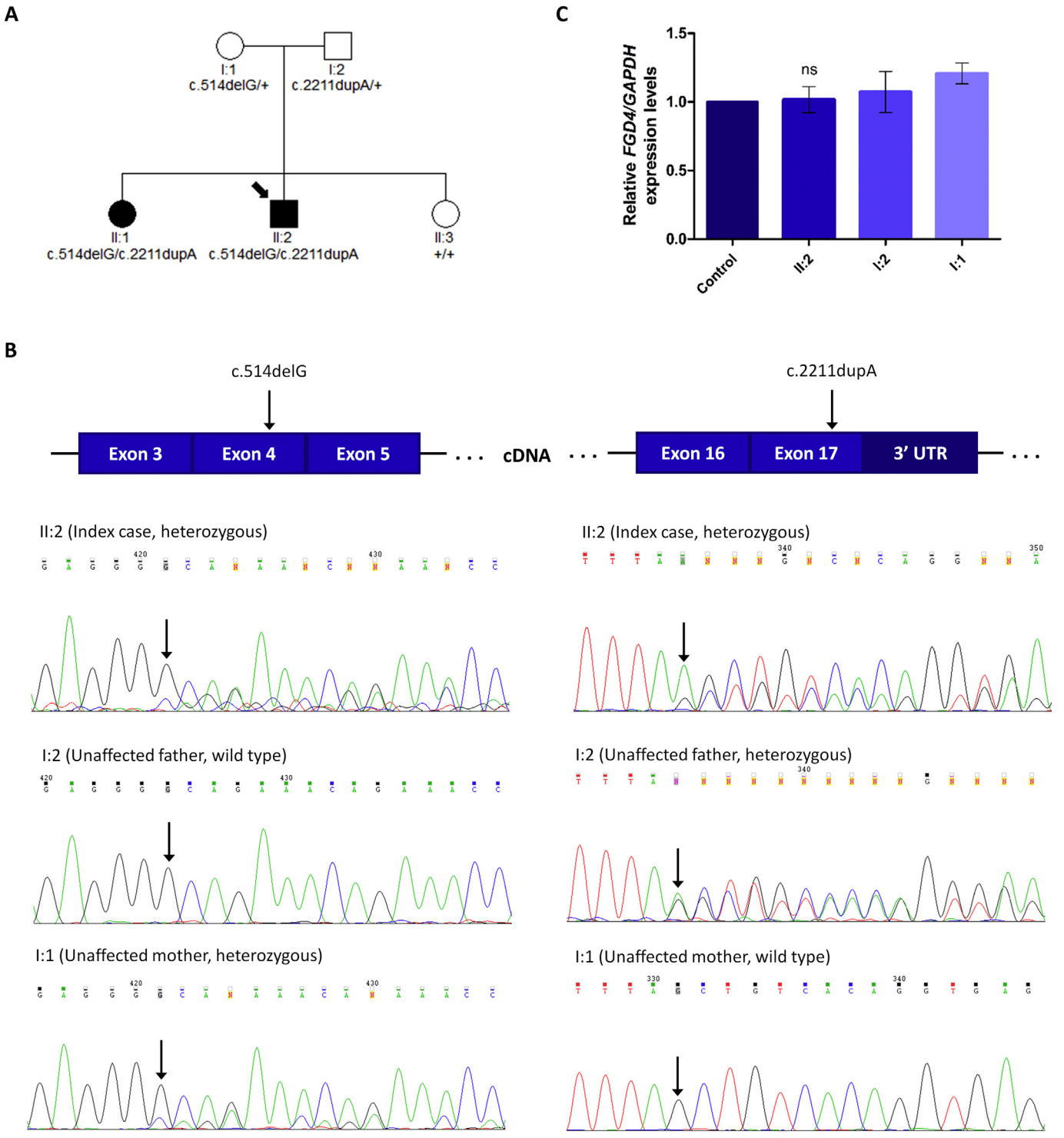

Fig. 2. Expression of the *FGD4* mutations in peripheral blood cells from the HUPLF965 family. A Family pedigree and *FGD4* genotypes. B Sequencing of the RT-PCR products obtained. Both mutations were evident in the cDNAs, as indicated by the arrow. C The qPCR analysis of *FGD4* mRNA from peripheral blood of the progenitors (I:1; I:2), index case (II:2) and the healthy control used as a calibrator. *GAPDH* was used as the reference gene for normalization and no significant (ns) differences were observed between index case II:2 and the rest of the samples (unpaired *t*-test, $p > .05$).

clinical phenotype of our patients, since they showed no signs of muscle atrophy or fat replacement. By contrast, muscle imaging in other patients with a mild phenotype revealed fat replacement in the anterior tibialis muscle [12], as well as atrophy of the anterior tibialis and hamstring muscles in a 10-year-old [9]. In our patients, NCVs were slow, a characteristic of CMT4H. However, their SNAPs were fairly well preserved, in contrast to the absence of SNAPs in the 31 nerve conduction studies reported previously, 26 of which were performed before or during adolescence [3,9–11,15–18].

Our genetic data indicate that neither mutation affects splicing or provokes the degradation of *FGD4* mRNA in the peripheral blood sample. Hence, each allele may produce a different truncated protein, although our analysis could not rule out whether or not these proteins may be degraded prematurely. Moreover, mRNA processing could be

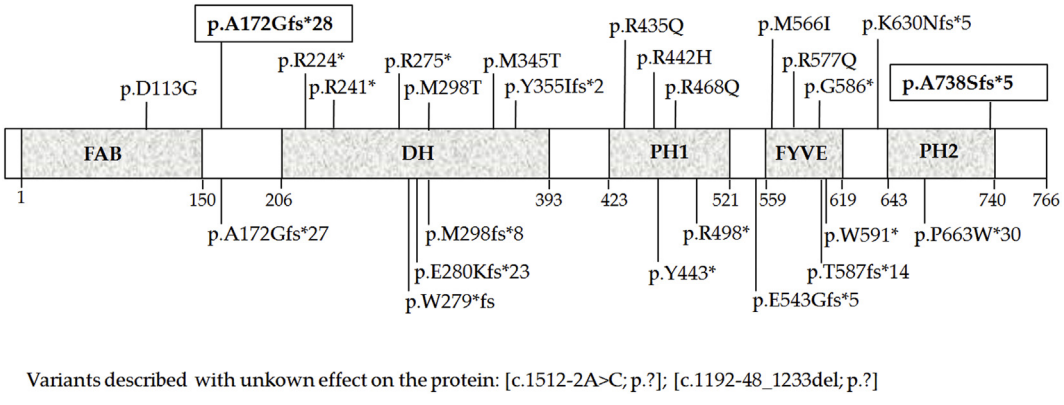

Fig. 3. Distribution of the pathological mutations reported in the *FGD4* protein. The mutations identified in our patients are indicated in bold and in boxes. Five functional domains are represented for *FGD4,* the FAB (F-actin binding), DH (Dbl homology), PH (Pleckstrin homology) and FYVE (Fab 1, YOTB, V ac 1, and EEA1 zinc finger) domains.

tissue-specific. Consequently, how these mutations affect splicing or degradation of mRNA in the peripheral nerve would still remain unknown. In lack of nerve tissue, investigation of mRNA processing and/or endogenous protein levels in other biological samples (i.e. from oral swab or skin biopsy) could be helpful. Considering the two truncated proteins predicted, the loss of functional domains in the p.Ala172Glnfs*28 protein means it is likely to be only weakly active at best, whereas the truncated p.Ala738Serfs*5 protein may partially have conserved *FGD4* activity since the main functional domains are retained. To date, the majority of *FGD4* variants are loss-of-function alleles that have been identified in homozygosity, and the mutations identified previously were at positions that differed from those in our patients p.Ala738Serfs*5 allele, lying more 5′ in the primary sequence. It is worth considering the p.Lys630Asnfs*5 variant in more detail, a variant found in homozygosis in three patients from two unrelated families: two siblings of Spanish origin [11] and a Turkish patient [18]. The Spanish siblings were said to have experienced symptoms from when they initiated independent walking [11], whereas the Turkish patient reported his first symptoms in the second decade of life. However, this latter individual experienced proximal weakness and cerebellar dysfunction when he was 28 years old, although clinical details about his phenotype are scarce [18]. The protein produced from the p.Lys630Asnfs*5 allele would lack part of the PH2 domain, whereas our patients' p.Ala738Serfs*5 allele would generate a truncated protein that maintains this domain. Indeed, the c.2211dupA variant produces a larger protein (p.Ala738Serfs*5), the largest truncated protein as yet described in CMT4H patients, which may be partially functional and hence explain the later onset and milder phenotype in our patients. Nevertheless, we cannot ignore the influence of environmental factors on gene expression (e.g., physical activity) and how the regular intense exercise undertaken by our patients could affect their clinical presentation.

5. Conclusions

The patients presented here carrying the c.514delG (p.Ala172Glnfs*28) and c.2211dupA (Ala738Serfs*5) mutations in *FGD4* had a very mild phenotype, as witnessed by electrophysiological and MRI examination. The in depth phenotyping and comprehensive genetic analysis carried out helps us to understand the pathogenic mechanisms associated with the different mutations and their influence on the final phenotype.

Acknowledgements

The authors wish to especially thank the patients and their family for their collaboration in this study. The authors acknowledge that the blood samples were processed, stored and delivered by La Fe Biobank. The authors thank the Instituto de Salud Carlos III (grant number PI16/00403), Generalitat Valenciana (grant number PROMETEO/2018/135), and the Health Research Institute Hospital La Fe (grant number 2017/0351) for their support.

Funding

This work was supported by the Instituto de Salud Carlos III (ISCIII, grant number PI16/00403 awarded to TS), and co-funded with FEDER funds and by the Generalitat Valenciana (grant number PROMETEO/2018/135 awarded to TS and CE). ASM received a contract supported by the Fundació Per Amor a l'Art and HAE was supported by the Health Research Institute Hospital La Fe (grant number 2017/0351).

Availability of data and materials

Please contact authors for data requests.

Authors' contributions

Conceived and designed the study: HAE, VL, TS. Clinical description and supervision of patients: HAE, MF, EMS, IP, MT. Performed the experiments and analysed the results: ASM, DMR, VL. Interpretation of the results: HAE, VL, CE, TS. Wrote the manuscript: HAE, VL, TS, with input from all other authors. All authors read and approved the final version of the final manuscript.

Ethics approval and consent to participate

This study was approved by Institutional Research Board of the Institute of Health Research, Hospital La Fe. All the protocols followed in this study complied with the ethics guidelines of the journal and the institutions involved. All patients and family members reported here gave their informed consent prior to commencing the study.

Consent for publication

Consent for publication was obtained from the patients and family members.

Competing interests

The authors have no conflict of interests to declare.

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