The *EGR2* gene is involved in axonal Charcot-Marie-Tooth disease

Teresa Sevilla[a,b,c], MD, PhD; Rafael Sivera[a], MD, PhD; Dolores Martínez-Rubio[b,d], MD, MS; Vincenzo Lupo[b,d], PhD; María J Chumillas[e], MD; Eduardo Calpena[b,d], MS; Joaquín Dopazo[b,f], PhD; Juan J Vílchez[a,b,c], MD, PhD; Francesc Palau[b,d], MD, PhD; Carmen Espinós[b,d,g], PhD.

[a]Department of Neurology, Hospital Universitari i Politècnic La Fe, Valencia, Spain.
[b]Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Valencia, Spain.
[c]Department of Medicine, University of Valencia, Valencia, Spain.
[d]Program in Genetics and Rare Diseases and IBV/CSIC Associated Unit, Centro de Investigación Príncipe Felipe (CIPF), Valencia, Spain.
[e]Department of Clinical Neurophysiology, Hospital Universitari i Politècnic La Fe, Valencia, Spain.
[f]Program in Computational Genomics, Centro de Investigación Príncipe Felipe (CIPF), Valencia, Spain.
[g]Department of Genetics, Universitat de València, Valencia, Spain.

*Corresponding author:
Dr. Carmen Espinós
Centro de Investigación Príncipe Felipe (CIPF)
Unit of Genetics and Genomics of Neuromuscular Diseases
c/ Eduardo Primo Yúfera, 3. 46012 Valencia. Spain.
E-mail: cespinos@cipf.es. Telephone no: +34 96 328 9680. Fax no.: +34 96 328 9701

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Abstract

Background and purpose: We investigated a three-generation family affected by axonal Charcot-Marie-Tooth (CMT) disease with the aim to discover the genetic defect and further characterize the phenotype.

Methods: We reviewed the clinical, nerve conduction and muscle magnetic resonance images of the patients. A whole exome sequencing (WES) was performed and the changes were investigated by genetic studies, *in silico* analysis and luciferase reporter assays.

Results: The novel c.1226G>A change (p.R409Q) in the *EGR2* gene was identified. Patients presented with a typical, late onset axonal CMT phenotype with variable severity that was confirmed in the ancillary tests. The *in silico* studies showed that the residue R409 is an evolutionary conserved amino acid. The p.R409Q mutation, which is predicted as probably damaging, would alter slightly the conformation of the protein and would cause a decrease of the gene expression.

Conclusions: This is the first report of an *EGR2* mutation presenting as an axonal CMT phenotype with variable severity. This study broadens the phenotype of the *EGR2*-related neuropathies and suggests that the genetic testing of patients suffering from axonal CMT should include the *EGR2* gene.

Introduction

Charcot-Marie-Tooth disease (CMT) refers to the heterogeneous group of inherited motor and sensory neuropathies. Molecular studies have shown an extensive genetic spectrum in CMT neuropathies with an ever-growing list of involved genes (http://neuromuscular.wustl.edu/time/hmsn.html), which render genetic screening increasingly challenging. In this regard, whole exome sequencing (WES) has become an efficient tool for genetic diagnosis.

Mutations in the *EGR2* gene result in a variety of demyelinating neuropathies in which severity ranges from severe congenital hypomyelinating neuropathy (CHN; MIM 605253) or Dejerine-Sottas syndrome (DS; MIM 1459000) to mild-moderate adult onset demyelinating CMT (CMT1D; MIM 607678). *EGR2* is required for myelination of the peripheral nervous system, it is activated in the Schwann cells before the onset of myelination and its disruption blocks Schwann cells at an early stage of differentiation [1]. The *EGR2* encodes for a zinc-finger transcription factor [2] that regulates myelin genes, such as *MPZ*, *Cx32/GJB1*, *PRX* and *PMP22* [1, 3-6].

We describe the clinical picture of five patients from the same family with axonal CMT (CMT2) harboring the novel *EGR2* p.R409Q mutation. To our knowledge CMT2 caused by *EGR2* mutations has not been previously reported and therefore, our findings broaden the spectrum of *EGR2*-related neuropathies.

Methods

Patients

We investigated a family (fCMT-248; Fig. 1A) with a probable autosomal dominant inheritance from our clinical series [7]. The clinical evaluation included a closed questionnaire about clinical symptoms and signs and an exhaustive physical

examination. The electrophysiological testing included sensory and motor nerve conduction velocities (MNCVs) registered with surface electrodes and standard needle electromyography with concentric needle [7]. Muscular MRI (magnetic resonance imaging) was performed, obtaining axial and coronal images of the feet, calves and legs; determining the presence of muscle oedema, muscle contrast enhancement, and fatty infiltration, grading this last with a semiquantitative scale from 0-4 [7, 8].

All protocols were approved by the Institutional Review Board of the Hospital Universitari i Politècnic La Fe. Written informed consents were obtained from all the studied individuals.

Genetic studies

Exome sequencing was performed in DNA from four patients (II:2, II:7, III:1 and III:13; Fig. 1A). Samples were subjected to exome enrichment with the Agilent SureSelect Human All Exon 50-Mb kit followed by sequencing using the Illumina HiSeq 2000 genome analyzer platform at CNAG (Centro de Análisis Genómico, Barcelona, Spain). The analysis was performed at the BIER platform (CIBERER) [9]. To investigate if the identified changes were SNPs (single nucleotide polymorphisms) different public databases were consulted: NCBI dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), Exome Variant Server, NHLBI Exome Sequencing Project (ESP) (http://varianttools.sourceforge.net/Annotation/EVS) and 1000 genomes (http://www.1000genomes.org/) databases.

The variants with uncertain significance were investigated by segregation analyses in the eleven available DNAs (Fig. 1A) and by mutational screening in healthy population. The Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/) was used to investigate the conservation of the candidate changes. The biological

relevance of the amino acid substitutions was studied using SIFT (http://blocks.fhcrc.org/sift/SIFT.html) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml) programs, and the effects of the changes were modelled using the program Coot (http://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/).

Plasmids, cell culture and reporter assay

A 400 bp fragment of the human *Cx32* promoter sequence containing the *EGR2* and *SOX10* binding sites was subcloned into the pGL2-basic luciferase reporter vector. The human *EGR2* (clon ID LIFESEQ8718902, OpenBiosystems, Thermo Scientific, Waltham, MA, USA) and mutants p.R409Q, p.R409W and p.R353W were subcloned into the pcDNA3 (Invitrogen, Carlsbad, CA, USA). *EGR2* mutants were obtained by PCR using the QuikChange site-directed mutagenesis kit (Invitrogen). Primers and PCR conditions are available upon request. Sanger sequencing confirmed all construct sequences.

HeLa cells were grown in DMEM medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% inactivated (v/v) fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen). Cells were cultured in 35-mm dishes and transfected with 1.8 µg of pGL2/Cx32-P, 0.150 µg of EGR wild-type, empty pcDNA3 or mutants plasmids, and 0.125 µg of pRL-TK using FuGENE HD (Promega, Madison, WI, USA). After 24 h cells were processed using the Dual-Luciferase® Reporter Assay System (Promega). Each plasmid combination was transfected in triplicate in every experiment.

Results

Clinical picture

The clinical characteristics are summarized in Table 1 and the nerve conduction and muscular MRI studies in Table 2. Symptom onset appeared after the twenties in all patients, but disease severity was quite variable.

The proband (II:7) was 52 years when first evaluated; she had gait abnormalities that started in her twenties and later involvement of distal hand muscles. MNCV of median nerve was 58 m/s and consequently the family was classified as CMT2. Clinical evaluation of her four children was normal, but the electrophysiological studies of individual III:13 (24 years) showed mildly decreased SNAP amplitudes with normal MNCV, and evidence of chronic denervation in distal lower limb muscles. At the time of evaluation he was 41 years and able to perform high altitude hiking, only referring slight difficulty when climbing. Clinical examination showed mild weakness in toe extension and loss of ankle reflex.

Patient II:2 was asymptomatic until her thirties, when she gradually developed gait abnormalities and progressive wasting and weakness of distal limb muscles. At age 67, examination showed upper and lower limb distal muscle wasting and weakness with proportional sensory loss. The follow-up was lost three years after, as she declined further medical visits due to increasing disability, but apparently weakness spread to proximal muscles, becoming wheelchair-bound at age 72. During the last two years of life, she had difficulty in swallowing and speaking, dying at age 82. Her son (III:1) was symptomatic from his twenties, having quite a fast progression. Clinical examination at age 56 showed important distal wasting and weakness in lower limbs, with decreased sensibility, needing ankle orthosis for walking.

Patient II:3 was evaluated at age 82. She was unable to pinpoint the onset of

symptoms but referred unsteadiness while walking for three or four decades. She had no symptoms in the upper extremities and current examination shows moderate weakness of ankle dorsiflexion, with normal hand strength.

Nerve conduction studies are consistent with a CMT2 form (Table 2). MNCV in upper limbs are consistently > 40 m/s in all patients except in subject III:1. Both motor and sensory amplitudes are reduced consistently with disease severity.

Needle EMG in all affected patients showed evidence of chronic denervation with spontaneous fibrillation potentials, large polyphasic potentials and reduced recruitment pattern in lower limb and distal upper limb muscles.

Clinical, nerve conduction studies and needle EMG in the healthy individuals III:2, III:9, III:11, and III:16 were normal.

MRI Studies

Intrinsic musculature of the feet showed consistent and bilateral fatty infiltration of the foot muscles in all cases, being proportional to severity. Varying degrees of fatty substitution in the calf muscles could also be detected, which were more prominent distally, in severely affected patients, and in the posterior (gastrocnemius > soleus) and anterolateral compartments (Table 2, Fig. 2). Muscle oedema was not observed in any of the studies. MRI of the thigh was performed in one patient (II:7), exhibiting a mild distal fatty infiltration of all the muscle compartments.

Genetic analyses

WES revealed that the four investigated patients (Fig. 1A) were heterozygous for the c.1226G>A substitution in *ERG2*, which is predicted to cause the amino acid change p.R409Q located in the third zinc-finger domain of the protein (Fig. 3). The search for

the *EGR2* c.1226G>A mutation in seven relatives (Fig. 1A) revealed that fully co-segregated with the disease. Moreover, this change was not observed in 214 healthy individuals of Spanish ancestry. Visualization of the 3D structure of the EGR2 protein (Protein Data Bank, PDB; entry P11161) showed that the p.R409Q substitution altered slightly its conformation (Fig. 1B). The computational analyses revealed that the R409 residue is an evolutionarily conserved amino acid and that the p.R409Q mutation is most likely damaging, with a SIFT score of 0.0 and a PolyPhen-2 score of 0.989. *In vitro*, the p.R409Q change decreased the transcriptional activity of the EGR2 protein to approximately 40% (Fig. 1C).

Discussion

We report the novel *EGR2* p.R409Q mutation in a family in which the age of onset and clinical characteristics resemble a 'classic' CMT2 phenotype with a variable rate of progression. It is striking that within the same family, a patient has developed quadriplegia, while others remain mostly asymptomatic. The two patients with quicker rate of progression are mother (II:2) and son (III:1), but no concomitant diseases or other mutations in CMT-related genes could be identified, as has been recognized in other kindred (Table 3) [10, 11].

Our proband (II:7) had MNCVs which were clearly in the axonal range as were the ones of II:2 and III:13, while patients II:3 and III:1 had slightly slower MNCVs, which could be described as being intermediate (Table 2). Very few other patients with *ERG2* mutations and MNCV around 40 m/s have been reported [3, 11, 12] but always in the setting of families with demyelinating CMT. These cases had a mild form of the disease when compared to other family members, whereas patient III:1 had important

disability when compared to those with greater MNCV. In the sequential nerve conduction studies performed in patient III:13, after 17 years of disease progression there was a decrease of CMAP predominantly in the lower limbs accompanied by a mild slowing of MNCV (Table 2).

The *EGR2* p.R409Q substitution was identified after performing WES in a large family in which most of CMT2 genes had already been ruled out by Sanger sequencing [7]. Subsequent genetic and computational studies support that the *EGR2* p.R409Q mutation is pathogenic. In the same codon, the p.R409W mutation has been described in a family with moderate CMT1 [3]. The p.R409Q change slightly alters the conformation of the protein while the p.R409W mutation probably destabilizes the protein (Fig. 1B). Despite the fact that the conformational change of the protein is more dramatic with the p.R409W mutation, *in vitro* the level of activation is decreased in a similar way to other previously described *EGR2* mutations like p.R359W and p.R409W (Fig. 1C), that are associated with CMT1D, DS and CHN [3, 10, 13, 14].

In reported clinical series, *EGR2* mutations represent about 1% of CMT1 [15], but has not been included in the standard mutational screening of CMT2 patients. Causative *ERG2* mutations have been published in 17 families and half of them correspond to *de novo* mutations associated with a severe phenotype (Table 3). All the disease-causing mutations fall within the three zinc-finger domains of the DNA-binding domain [16, 17], except the p.I268N change (Fig. 3) transmitted as an autosomal recessive mutation [3]. *EGR2* mutations exert a dominant-negative effect on the activation of some endogenous target genes including myelin associated genes, especially *MPZ* [5, 18, 19]. Taking this into account it seems plausible that as more mutations in *EGR2* are described, the clinical spectrum will resemble that of the *MPZ* mutations, which can cause CHN, DS, and CMT1 phenotypes, but also adult onset

axonal-intermediate CMT.

MPZ is the major structural protein of peripheral nervous system myelin, and the molecular pathways by which certain mutations in *MPZ* can cause primary axonal degeneration with minimal myelin involvement remain to be completely understood. In these cases the protein retains its cellular localization, but there is a reduction in its adhesiveness [20]. In a postmortem study 'aggresomes' were found in the periaxonal space, with important reorganization of the molecular architecture of the axolemma [21]. These alterations are likely to disrupt the axon-myelin interplay necessary for the maintenance of axonal structure and transport [22]. A similar physiopathologic process can be observed in certain mouse models with deficient myelin or glial proteins, like myelin associated glycoprotein (MAG), or 2,3-cyclic nucleotide phosphodiesterase (Cnp1), which develop axonal degeneration with preserved myelin [23, 24]. It is tempting to speculate that the mild conformational changes caused by the *EGR2* p.R409Q mutation permit an adequate myelination, but ultimately interrupt the axon-myelin interactions necessary for axon maintenance.

This is the first report of a novel *EGR2* mutation causing a late onset CMT2 with variable severity. The characterization of this new phenotype illustrates the broad spectrum of *EGR2*-related neuropathies and is relevant in order to gain insight into the pathomechanism of the disease.

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FIGURE LEGENDS

Figure 1: (A) Pedigree of family CMT-248. Exome of individuals II:2, II:7, III:1 and III:13 was sequenced. Available DNAs are marked with an asterisk. (B) Visualization of the effect of p.R409Q in the EGR2 protein. From left to right: native structure; effect of p.R409Q; and effect of p.R409W. (C) Histograms representing the percentage of activation of *EGR2*. The 100% was assumed for *EGR2* and all the others are referred to this maximal value (**$p<0.01$).

Figure 2: Axial muscular magnetic resonance T1-wheighted images of patients III:13 (A), II:7 (B) and II:3 (C). Fatty streaks in the gastrocnemius and lateral compartment of the calf (A) in scarcely symptomatic patient III:13. Predominance of fatty infiltration in the posterior and anterolateral compartments of the calf in more severely affected patients (B, C).

Figure 3: Reported mutations distributed along the *EGR2* gene. All of them are within the zinc-finger domains, except p.I268N, described in homozygosis in three siblings suffering from CHN [3]. The position of the domains is according to UniProt (entry P11161).

Table 1 Clinical features of patients belonging to family fCMT-248.

Patient	Sex	Onset/First symptom	Initial evaluation			Current state					
			Age	UL/LL weakness	Sensory loss	Age	UL/LL weakness	Sensory loss	CMTNS	CMTES	Disability
II:2	F	30 y, LL weakness	67 y	++/+++	Yes	82 y[a]	NA	NA	NA	NA	Dead at 82 y, quadriplegia and cranial nerve palsies at 80 y
II:3	F	50 y, Awkward gait	82 y	-/+	Yes	82 y	-/+	Yes	7	6	Unable to run
II:7	F	20 y, LL weakness	52 y	+/+	Yes	72 y	+/++	Yes	12[a]	10	Steppage gait, unable to walk quickly
III:1	M	20 y, LL weakness	35 y	+/++	Yes	56 y	+/+++	Yes	20	17	Needs a cane for support
III:13	M	Asymptomatic	24 y	No	No	41 y	-/+	No	6	3	Mild weakness in toes; normal mobility

F, female; M, male; LL, lower limb; UL, upper limb; CMTES, CMTNS, Charcot-Marie-Tooth neuropathy score; Charcot-Marie-Tooth examination score; +, mild distal weakness; ++, moderate distal weakness; +++, severe distal weakness or proximal involvement; NA, not applicable. [a]CMTNS in this patient was calculated with current clinical scores and previous nerve conduction studies (at age 52).

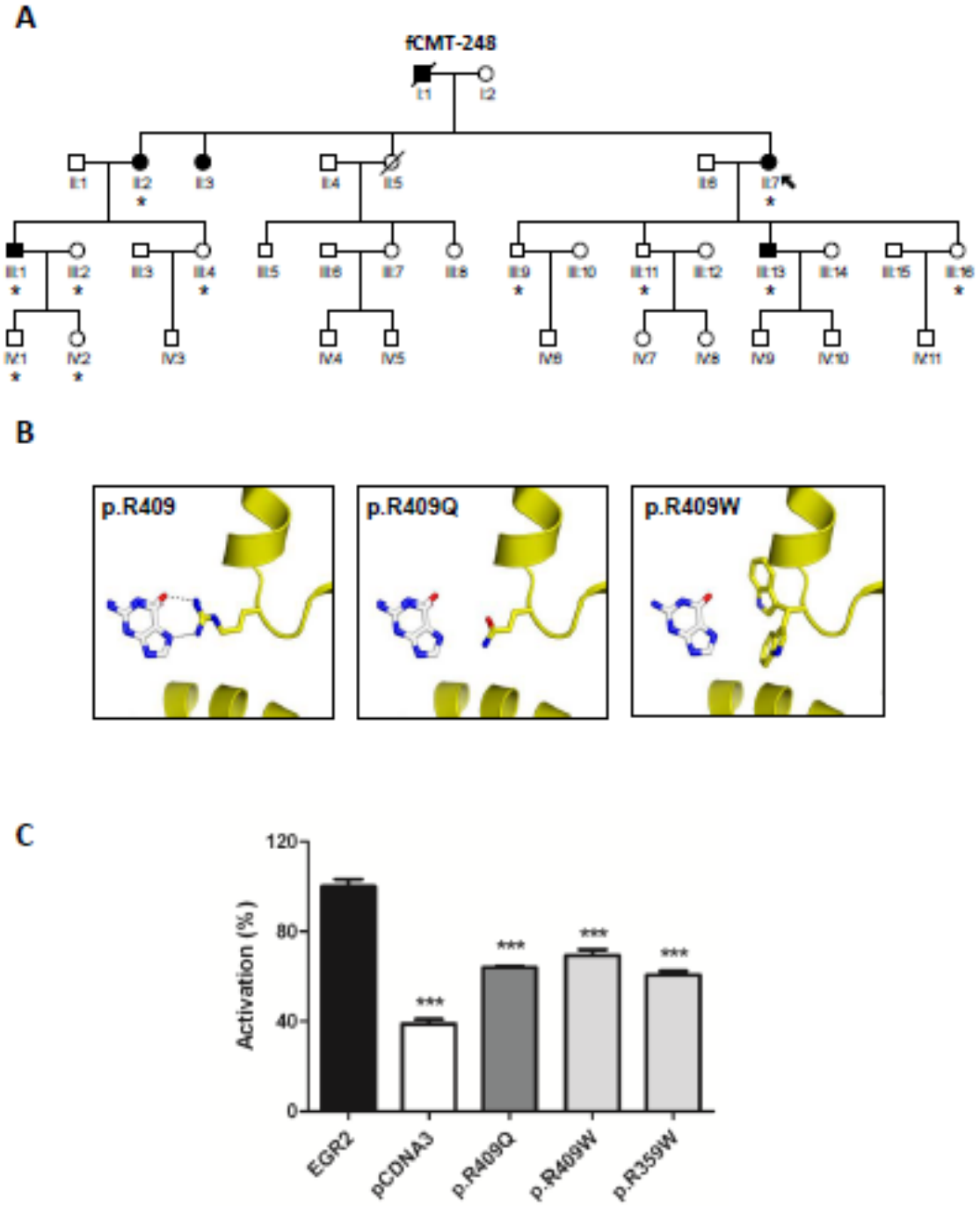
A
fCMT-248
I:1
I:2
II:1
II:2
II:3
II:4
II:5
II:6
II:7
II:8
II:9
II:10
II:11
II:12
II:13
II:14
II:15
II:16
IV:1
IV:2
IV:3
IV:4
IV:5
IV:6
IV:7
IV:8
IV:9
IV:10
IV:11
B
p.R409
p.R409Q
p.R409W
C
Activation (%)
120
80
40
0
***
***
***
***
EGR2
pCDNA3
p.R409Q
p.R409W
p.R359W

Figure 1.

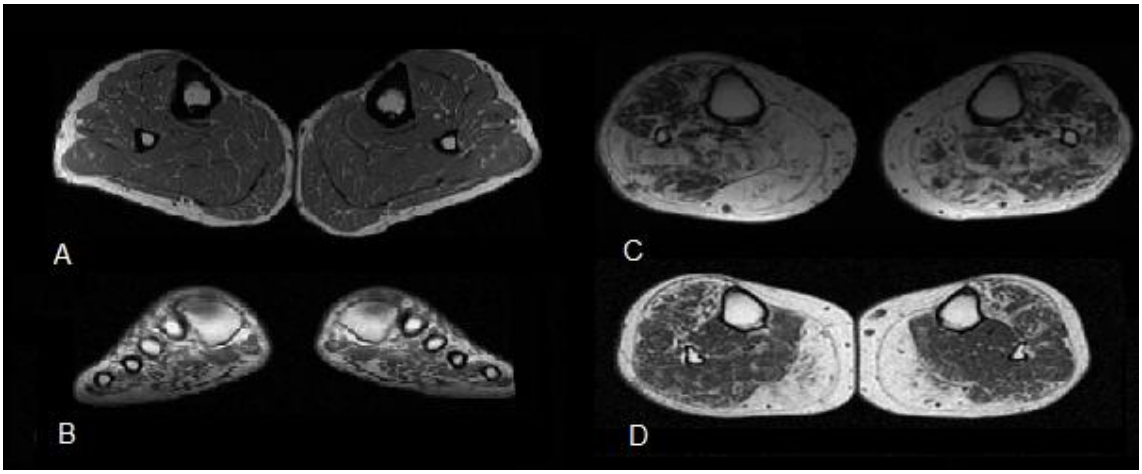

Figure 2.

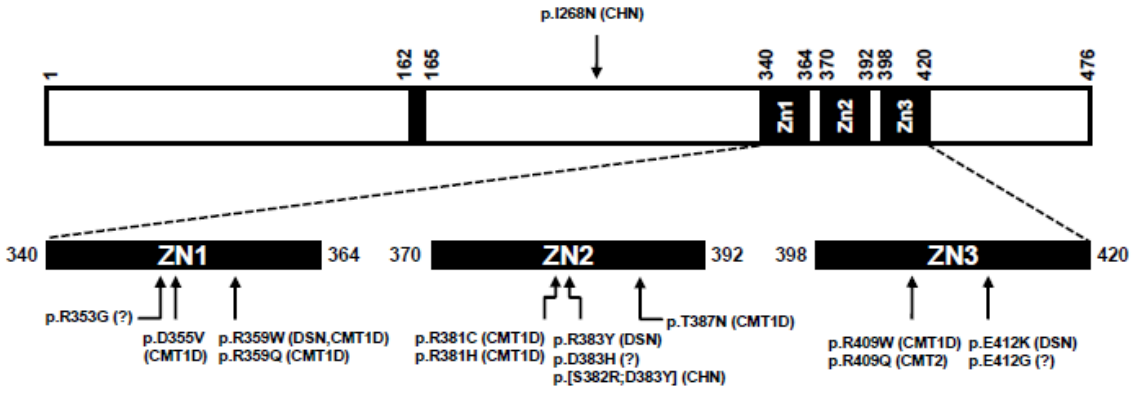

Figure 3.