ONLINE MUTATION REPORT

Frequency of mutations in the early growth response 2 gene associated with peripheral demyelinating neuropathies

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Hereditary motor and sensory neuropathies (HMSN) comprise a wide clinical spectrum of related disorders with defects in peripheral nerve myelination. More than 250 distinct mutations of the peripheral myelin protein 22 (*PMP22*), myelin protein zero (*MPZ/P0*), and connexin 32 (*Cx32/GJB1*) genes have been reported in patients diagnosed with different forms of hereditary motor and sensory neuropathies, such as Charcot-Marie-Tooth disease (CMT), Dejerine-Sottas syndrome (DSS), congenital hypomyelination (CH), and hereditary neuropathy with liability to pressure palsies (HNPP). To date, some 20 linked CMT loci and nine genes have been identified. Gene products include structural proteins (*PMP22*, *MPZ*), a gap junction protein (*Cx32*), and a transcription factor (*EGR2*). Recently, point mutations in the early growth response 2 (*EGR2*) gene, located on human chromosome 10q21.1-q22.1, have been associated with hereditary neuropathies.¹

The gene for *EGR2* spans 4.3 kb, contains two coding exons,² and is part of a multigene family encoding Cys,His₂ type zinc finger proteins³ and may play a role in the regulation of cellular proliferation.⁴ *EGR2* is a Schwann cell transcription factor that binds DNA through three zinc finger domains and is thought to regulate the expression of late myelin genes such as *MPZ* and myelin basic protein, thus playing a key role in myelogenesis.⁵

Human *EGR2* is homologous to the mouse *Krox20* gene,⁶ which is part of a transcriptional cascade involved in the development and segmentation of the hindbrain. Homozygous mice knocked out for *Krox20* display abnormal rhombomere segmentation and neuronal migration in the developing hindbrain, resulting in anatomical abnormalities of the cranial nerves.⁷ Furthermore, mice homozygous for a targeted mutation deleting a major part of the gene including the entire zinc finger domain die shortly after birth.⁸ Surviving *Krox20*^{-/-} mice show a trembling phenotype, have hypomyelination of the peripheral nervous system, and their Schwann cells are blocked at an early stage of differentiation. Heterozygous *Krox20*^{+/-} mice have no functional impairment or neuropathological abnormalities.⁵

To date, eight different *EGR2* mutations have been found in patients with clinical features of CH, CMT1, or DSS.¹⁻¹⁴

We examined a collection of 101 patients, some with well established diagnostic criteria, and we also included a group with little or no clinical information. Our aim was to document the frequency of *EGR2* mutations in the CMT population.

We report a heterozygous de novo missense mutation in the *EGR2* gene in a patient diagnosed with CH associated with cranial nerve deficits. This mutation was previously presented with preliminary data in abstract format¹⁵ and is now documented in detail. Furthermore, we identified three different, probably silent exonic polymorphisms in the *EGR2* transcription factor.

Key points

- A panel of 101 patients with different clinical features of Charcot-Marie-Tooth disease was screened for mutations in the *EGR2* gene.
- The presence of the CMT1A duplication and mutations in the *GJB1*, *PMP22*, and *MPZ* genes were preliminarily excluded.
- Only one mutation in a zinc finger domain of *EGR2* was found in a patient with congenital hypomyelination. Three other sequence variants were found not directly associated with the disease.
- The frequency of mutations in the *EGR2* gene is estimated to be less than 1% among CMT patients.

MATERIALS AND METHODS

Selection and phenotypical classification of the patients

One hundred and one unrelated probands presenting with peripheral neuropathy and without *PMP22*, *MPZ*, or *GJB1* mutations¹⁶⁻¹⁸ were studied from the laboratory collections of Lyons (51 patients) and Cardiff (50 patients). They all presented with at least weakness and progressive wasting of the distal limb muscles, pes cavus, and absence of deep tendon reflexes.

The criteria for the classification of different conditions included:

- Congenital hypomyelination: hypotonia, delayed motor milestones, biopsy showing absence of myelination, NCV less than 10 m/s.¹⁹
- Dejerine-Sottas syndrome: isolated CMT cases compatible with autosomal recessive transmission, NCV less than 10 m/s, onset in childhood. Biopsy, when available, must show demyelination.²⁰
- CMT1: NCV between 15 and 35 m/s associated with typical CMT phenotype.²¹⁻²²
- Unspecified phenotype: with the presence of only one bit of clinical information, either NCV or transmission mode.

Mutation analysis

The two exons of the *EGR2* gene were analysed using nine overlapping primer sets based on the cDNA sequence.¹

The coding region of *EGR2* was amplified using 200 ng of genomic DNA, 0.5 U *Taq* polymerase (Euroblutaq® DNA polymerase, Labo Eurobio, France), 2.5 µl 10 × PCR buffer, 0.75 µl 50 mmol/l MgCl₂ solution, 2 µl of each primer at a stock concentration of 20 mmol/l, 1 µl 10 mmol/l dNTPs, and H₂O to a total volume of 25 µl.

Amplification conditions were denaturation for one minute at 94°C, followed by 35 cycles of denaturation at 94°C for one

Table 1 Information on the 101 patients selected for *EGR2* study

Phenotypic classification	Origin		Sex		Total
	Lyon	Cardiff	M	F	
Hypomyelination	5	0	0	5	5
DSS	13	0	12	1	13
CMT1	33	25	23	35	58
Unspecified phenotype	0	25	14	11	25

DSS=Dejerine-Sottas syndrome, CMT1=Charcot-Marie-Tooth type 1, M=male, F=female.

minute, annealing at the optimal temperature for each primer pair, and elongation at 72°C for one minute using the T Gradient Whatman Thermocycler 1999 (Biometra®, Goettingen, Germany). The PCR product was labelled with 1 µCi (0.1 µl) of [α -³²P]dCTP or [α -³³P]dCTP (Amersham Pharmacia Biotech, UK) in the reaction mixture.

For SSCP analysis, 5 µl of amplified DNA was mixed with 3 µl formamide sample buffer, denatured for five minutes at 100°C, and separated on a 25% Hydrolink MDE (BMA Biowhittaker Molecular Applications, Rockland, ME) gel at 12 W for 17 hours at room temperature. Some mutations were only detected by electrophoresis in the additional presence of 4% glycerol. Gels were vacuum dried and autoradiographed for four hours at -70°C. Proband in whom restriction sites in the *EGR2* gene were destroyed or created were screened by restriction enzyme digestion applying the manufacturer's instructions. The R362R polymorphism (CGA→CGC) of codon 362 destroys a restriction site for the *HinfI* enzyme (New England Biolabs, Beverly, MA). Twenty-five µl of PCR product were digested with 20 U of *HinfI* in a total volume of 50 µl. Samples were incubated at 37°C for 17 hours and analysed on 2% agarose gels. The polymorphism was further confirmed by sequencing. The presence of the *HinfI* polymorphism was tested in 164 normal control samples.

Sequencing

The PCR products were purified (QIAquick PCR Purification Kit, Qiagen SA, Courtaboeuf, France) and sequenced using dideoxy terminator technology (PRISM™ Ready Reactions Rhodamine Terminator reagent set; Applied Biosystems, Foster City, CA) and an automated sequencer (ABI Prism 310, Applied Biosystems, Perkin Elmer).

RESULTS

Classification of phenotype

The 101 patients (49 men, 52 women) with HMSN were classified in accordance with criteria described in the Materials and methods section. When clinical and paraclinical information was available, distinction was made between DSS, CH, and CMT1 (table 1). All patients had been screened and were negative for the CMT1A duplication and for mutations in *GJB1*, *PMP22*, and *MPZ*. The 33 CMT1 patients from the Lyon laboratory are familial cases with dominant transmission.

Mutations/polymorphisms

Nine patients had an altered SSCP pattern for *EGR2* exon 2.

Patient T58 presented with an insertion of a triplet (A300-301ins: c.900-902insGCA), found in amplicon 2.4, coding for alanine at position 300, a region which contains a repetition of 10 alanines, upstream of the first zinc finger. T58 was a 23 year old male with distal and proximal muscle wasting and weakness. No affected subjects were present in preceding generations. However, this insertion in the coding sequence was not detected in the proband's 25 year old sister who presented with the same clinical features. This sequence variation was found only once in our collection of 101 patients and in 70 controls.

Patient LY766.3 presented with a missense mutation in codon 381 (R381H: c.1142G>A), found in amplicon 2.5b, which is located in the second zinc finger domain and leads to an amino acid substitution of His for Arg. This patient was a 9 year old girl with no family history of any neuromuscular disorder and her parents were not consanguineous. Her four brothers were healthy on clinical diagnosis. The patient's birth was normal but she presented with congenital hypotonia. Walking was acquired at 20 months. Very soon, left Duane syndrome (hypoplasia of the VIth cranial nerve, strabismus) was noted. Clumsiness was noted at the age of 3 at nursery school. The distal motor deficit was evident at the age of 5 in the lower limbs and at 7 in the upper ones. Right nystagmus was noted at the age of 8. Now, at the age of 9, the deficit is reaching the proximal part of the limbs, and sensory abnormalities are appearing in the distal part of the legs. The motor NCVs of the median nerve ranked between 7 and 14 m/s. This patient was classified in the CH group, despite the lack of a biopsy.

The R381H mutation was not present in the healthy parents of patient LY766.3, indicating that this is a de novo *EGR2* mutation. This *EGR2* mutation was absent in 70 normal control samples.

A silent mutation in codon R362 of the third base (R362R: c.1086A>C), found in amplicon product 2.5c, was detected in six patients (6%), once in a DSS patient and five times in CMT1 patients. This mutation destroys a *HinfI* restriction site. The same mutation was present in two of 164 normal control samples (0.6%).

Patient T14 with a CMT1 phenotype presents with a deletion of nucleotides 17, 18, and 19 downstream from the stop codon, found in PCR amplicon 2.6 of the *EGR2* gene (c.1442-1444delATA).

DISCUSSION

Patient LY766.3 is part of a selected group of 18 patients with a severe phenotype and who presents with a heterozygous missense mutation in codon 381.

Cranial nerve involvement associated with R381H mutation may be related to the role of Krox20/*EGR2* in brainstem development, because involvement of different cranial nerves have been rarely reported.²⁴⁻²⁵ Although oculomotor nerve palsy has not been reported before in DSS and CMT patients, a subclinical deficit cannot be excluded. The first two described cases of the R359W mutation with DSS were also associated with clinical cranial nerve involvement.^{10 11}

It predicts an R381H substitution within the alpha helix of the second zinc finger. The DNA binding properties of the transcription factor are localised in the zinc finger domains and interact with DNA at the consensus *EGR2* binding site (5' GCGTGGGCG 3').²² For DNA binding, the arginines are able to form hydrogen bonds on the G rich strand of the consensus binding site.²³ These hydrogen bonds are most likely not established when the arginine residue is mutated to a histidine, suggesting that R381H is a loss of function mutation.¹¹ The phenotype is probably the result of an altered interaction between *EGR2* and the DNA binding sequence, since the protein sequence is highly conserved in that region.

Table 2 Previously reported *EGR2* mutations

No [Ref]	Mutation	Phenotype
1 [1]	c.1225C>T R409W substitution 3rd zinc finger	Familial CMT1 case with autosomal dominant transmission
2 [1]	c.803T>a I268N substitution inhibitory domain R1	3 affected sibs (four boys) from consanguineous marriage with CH
3 and 4 [6,1]	c.[1146T>G + 1147G>T] S382R and D383Y substitution 2nd zinc finger	Patient with sporadic CH
5 [9]	c.1064A>T D355V substitution 1st zinc finger	Severe CMT1 case (de novo mutation)
6 [10,11,14]	c.1075C>T R359W substitution α -helix 1st zinc finger	3 cases with DSS (new mutations)
7 [13]	c.1141C>T R381C substitution 2nd zinc finger	Familial moderate severe CMT1 case with autosomal dominant transmission
8 [12]	c.1142G>A R381H substitution 2nd zinc finger	Familial CMT1 case with cranial nerve involvement (III, IV, V, VII, VIII, X, XI)

To date, eight different mutations have been found in *EGR2* (table 2). The phenotypes in relation to mutations which fall within the region encoding a zinc finger domain are likely to be the result of the altered DNA binding properties of the transcription factor. The variation in clinical severity observed with the zinc finger mutations appears to correlate with the level of residual DNA binding.⁷

The most severe phenotype is associated with the mutant which confers the greatest level of DNA binding and transcriptional activation, while the least severe phenotype is observed in the mutant which shows no binding or transcriptional activity.⁷ This may explain the fact that DNA binding might be less affected by substitution of the cysteine residue than the histidine residue.¹³

Six patients (6%) of our series had the R362R silent mutation (CGA→CGC) but the same polymorphism was found twice in 164 normal control samples. In another study,¹¹ the same silent heterozygous mutation has been reported once in a patient with demyelinating neuropathy, but also once in 70 normal control samples. The frequency of this polymorphism in the general population should be about 2.5%.

The insertion of GCA, coding for alanine in position 300 of the *EGR2* protein, is situated in a repeated sequence of 10 alanines, upstream of the first zinc finger. The insertion of the triplet does not disturb the open reading frame and there is no creation of a potential supplementary splice site as tested by a splice site prediction computer program.²⁷ The repeated sequence forms an alpha helix, upstream of the first zinc finger domain. In mouse, the domain is partially conserved but is three alanines shorter. A modification of secondary structure in this region could be responsible for loss of function of the *EGR2* protein.

Codon 300 falls within a probable R1 inhibitory domain. Does the insertion of an alanine abolish or perturb the interaction with corepressors NAB1 and NAB2 which are known to increase the transcriptional activity of *EGR2* as has been shown for the I268N mutation? The proband's sister, however, shows the same phenotype and does not show the heterozygous insertion. We can thus deduce that the insertion probably has no pathological effect. We tested 70 normal control samples, none of which showed the polymorphism. We can conclude that the insertion is a very rare variant, with or without a minor effect on secondary structure of the R1 inhibitory domain of *EGR2*.

The patient who has deletion of three nucleotides with positions 17, 18, and 19 immediately following the stop codon

has a CMT1 phenotype. Downstream of the termination codon, the primary transcript is cleaved some 15-30 nucleotides after a polyadenylation signal. In *EGR2*, the polyadenylation signal is located 1180 nucleotides beyond the termination codon.³ It is unlikely that the present deletion in some way affects the correct cleaving and addition of the poly(A) tail. Although not found in the 100 other patients of our series or in 70 normal controls, this is probably a very rare sequence variant. No relatives of this patient were available for analysis.

The fact that the CMT, CH, and DSS phenotypes are associated with mutations of the same gene has previously been observed for *MPZ* and *PMP22*. Altered expression of a dosage sensitive gene in the peripheral nervous system can modify myelin structure and could potentially cause CH, in much the same way that increased *PMP22* expression owing to the *CMT1A* duplication results in CMT1.²⁸ Discordance between *PMP22* point mutations in humans and mice with both a severe phenotype and a mild phenotype produced by *PMP22* +/- (heterozygous deletion of the gene) underlines the important fact that the partial deletion of *PMP22* seems less deleterious than a point mutation. This finding suggests that point mutations in *PMP22* are not responsible for simple loss of gene function but represent a toxic gain of function.²⁹

Rather than acting as loss of function alleles, zinc finger mutations may instead be acting as dominant negative or gain of function alleles either by forming inappropriate interactions with cofactors, or by binding to inappropriate targets. Elucidation of the exact pathomechanism underlying these zinc finger mutants will require the identification of the relevant target genes in Schwann cells. Research showed no direct Krox20 binding to the *MPZ* promoter,³⁰ although this study showed that Krox20 is capable of transactivating the *MPZ* promoter, but did not indicate whether it does so directly or indirectly. Sequence similarity searches for the *EGR2* consensus binding site in the known promoters of *PMP22* and *GJB1*, the other two myelin specific genes known to be involved in this group of peripheral neuropathies, have not shown any potential *EGR2* binding sites.³¹

The specificity of the phenotype may reflect tissue specific interactions among the various constituents of the transcriptional machinery, complementation by the different members of the *EGR2* family in the other involved systems, and/or sensitivity of the PNS myelin to changes in myelin dosage (like in *PMP22*).³²

In conclusion, we confirm that *EGR2* mutations are not frequent in HMSN and represent <1% of the cases. Their occurrence seems restricted to the more severe phenotypes of DSS

and CH (seven out of nine cases reported so far) but they are also, more rarely, associated with a CMT1 phenotype.

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