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

N Vandenberghe, M Upadhyaya, A Gatignol, L Boutrand, M Boucherat, G Chazot, A Vandenberghe, P Latour

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 palsy (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 16q21.1-q22.1, have been associated with hereditary neuropathies.

The gene for EGR2 spans 4.3 kb, contains two coding exons, and is part of a multigene family encoding Cys-His-type zinc finger proteins and may play a role in the regulation of cellular proliferation. 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.

Human EGR2 is homologous to the mouse Krox20 gene, which is part of a transcriptional cascade involved in the development and segmentation of the hindbrain. Heterozygous mice knocked out for Krox20 display abnormal rhombomere segmentation and neuronal migration in the developing hindbrain, resulting in anatomical abnormalities of the cranial nerves. Furthermore, mice homozygous for a targeted mutation deleting a major part of the gene including the entire zinc finger domain die shortly after birth. 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.

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

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 format 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 PMP22, MPZ, and GJB1 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 mutations 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.
- 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.
- CMT1: NCV between 15 and 35 m/s associated with typical CMT phenotype.
- 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. The coding region of EGR2 was amplified using 200 ng of genomic DNA, 0.5 U Taq polymerase (Eurobluetaq® 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
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 (Biometa®, Geottingen, Germany). The PCR product was labelled with 1 μCi (0.1 μl) of (α-32P)dCTP or (α-32P)dATP (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 tech, UK) in the reaction mixture. The 362R polymorphism (CGA→GGC) of exon 362 destroys a restriction site for the Hinfl enzyme (New England Biolabs, Beverly, MA). Twenty-five μl of PCR product were digested with 20 U of Hinfl 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 Hinfl 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 GB1, DMP22, 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 ampiclon 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 exon 381 (R381H: c.1142G>A), found in ampiclon 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 Vth 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 exon 362 of the third base (R362R: c.1086A>C), found in ampiclon product 2.5c, was detected in six patients (6%), once in a DSS patient and five times in CMT1 patients. This mutation destroys a Hinfl 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 ampiclon 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 exon 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. 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 R335W mutation with DSS were also associated with clinical cranial nerve involvement.

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′ GGCTGGGGCGG 3′). For DNA binding, the arginines are able to form hydrogen bonds on the G rich strand of the consensus binding site. 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. 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>
<thead>
<tr>
<th>Phenotypic classification</th>
<th>Origin</th>
<th>Lyon</th>
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<th>M</th>
<th>F</th>
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</tr>
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<td>25</td>
<td>14</td>
<td>11</td>
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<td>50</td>
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</table>

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

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. This may explain the fact that DNA binding might be less affected by substitution of the cysteine residue than the histidine residue.

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, 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 alanines, upstream of the first zinc finger. The insertion of the EGR2 protein, is situated in a repeated sequence of 10

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

Table 2 Previously reported EGR2 mutations

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

ACKNOWLEDGEMENTS

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