Giant axonal neuropathy: clinical and genetic study in six cases

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Background: Giant axonal neuropathy (GAN) is a severe recessive disorder characterised by variable combination of progressive sensory motor neuropathy, central nervous system (CNS) involvement, and "frizzy" hair. The disease is caused by GAN gene mutations on chromosome 16q24.1

Aims: To search for GAN gene mutations in Turkish patients with GAN and characterise the phenotype associated with them.

Methods: Linkage and mutation analyses were performed in six affected patients from three consanguineous families. These patients were also investigated by cranial magnetic resonance imaging (MRI) and electroencephalography (EEG). Electromyography (EMG) was performed in heterozygous carriers from family 1 and family 3.

Results: Linkage to 16q24.1 was confirmed by haplotype analysis. GAN mutations were identified in all families. Family 1 had the R293X mutation, previously reported in another Turkish family. Families 2 and 3, originating from close geographical areas, shared a novel mutation, 1502+1G>T, at the donor splice site of exon 9. All patients displayed a common phenotype, including peripheral neuropathy, cerebellar ataxia, and frizzy hair. Cranial MRI showed diffuse white matter abnormalities in two patients from family 1 and the patient from family 3, and minimal white matter involvement in the patient from family 2. EMG of a heterozygous R293X mutation carrier showed signs of mild axonal neuropathy, whereas a 1502+1G>T mutation carrier had normal EMG. EEG abnormalities were found in three patients.

Conclusion: These findings highlight the association of CNS involvement, in particular white matter abnormalities, with peripheral neuropathy in GAN. The phenotypical consequences of both mutations (when homozygous) were similar.

Giant axonal neuropathy (GAN; MIM 256850) is a recessive neurodegenerative disorder affecting both the peripheral nervous system and the central nervous system (CNS). Neurological abnormalities are usually associated with a characteristic “frizzy” hair abnormality, although several patients with normal hair structure have been reported. A Tunisian form of GAN with normal hair, associated with a characteristic "frizzy" hair abnormality, motor neurone degeneration was also described. Electron microscopic examination of axonal swellings in peripheral nerves revealed excessive accumulation of neurofilaments in GAN. An aggregation of cytoplasmic intermediate filaments was also seen in other cell types, including fibroblasts, endothelial cells, melanocytes, and Langerhans cells, indicating a generalised disorganisation of cytoplasmic intermediate filaments in GAN. We and others have located the GAN disease locus to 16q24.1 by homozygosity mapping in consanguineous families, and we have recently identified the defective gene by positional cloning. The interaction of gigaxonin with MAP1B-LC enhances the microtubule stability required for axonal transport. Moreover, microtubule destabilisation has been shown to induce intermediate filament aggregation in GAN fibroblasts, suggesting a role for gigaxonin in intermediate filament and microtubule networks.

SUBJECTS
Three consanguineous families were investigated in our study (fig 1). All families gave informed consent. The clinical diagnosis of GAN was made on the following criteria: progressive sensory motor peripheral neuropathy; features of CNS involvement such as mental retardation, cerebellar abnormalities, pyramidal tract signs, and cranial nerve abnormalities; frizzy hair; electromyography (EMG) findings of axonal neuropathy; and ultrastructural findings of increased cytoplasmic intermediate filaments in sural nerve or skin specimens. A sural nerve biopsy from the index patient in family 1 (patient 1) and skin biopsies from patient 2 in family 1 and patient 5 in family 2 were obtained. No biopsy sample was available from patient 6 in family 3. The diagnosis of GAN was confirmed by linkage and mutation analyses in these families.

Abbreviations: EEG, electroencephalography; EMG, electromyography; GAN, giant axonal neuropathy; MAP1B, microtubule associated protein 1B; MAP1B-LC, microtubule associated protein 1B light chain; MNCV, motor nerve conduction velocity; MRI, magnetic resonance imaging; PCR, polymerase chain reactions; SSCP, single strand conformational polymorphism.
based solely on clinical grounds for this patient. Direct physical examination of two affected siblings in family 1 (individuals (III)-1 and (III)-2) was not possible, but they were reported to have distal limb weakness, truncal ataxia, mental deterioration, frizzy hair, and skeletal deformities, resembling phenotypically the other patients in the same family. Heterozygous carriers displayed none of the clinical features of GAN and their physical examination was normal. All families originated from southeastern Turkey.

**METHODS**

**Electrophysiological and neuroimaging studies**

EMG and electroencephalography (EEG) were performed in four patients and six patients, respectively. EMG was also carried out in two clinically unaffected heterozygous carriers (individual (II)-14 in family 1 and individual (I)-1 in family 3). Other heterozygous carriers did not consent to EMG. Patients 1, 2, 5, and 6 underwent cranial magnetic resonance imaging (MRI). MRI examinations were performed on 1.5 T systems. Cranial MRI examinations included T1 and T2 weighted axial and coronal images.

**Histological and ultrastructural analyses**

**Nerve biopsy**

Sural nerve samples were obtained from 5–6 cm above the lateral malleolus. One part of the nerve was snap frozen in liquid nitrogen and 12 μm frozen sections were stained with haematoxylin and cosin and modified Gomori trichrome. The
rest of the specimen was fixed in 3% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated through serial alcohol baths, and embedded in Spurr’s resin. Semithin sections for light microscopy were stained with toluidine blue. Ultrathin sections were contrasted with uranylacetate and lead citrate and examined under a Zeiss 952 electron microscope.

Skin biopsy
Skin specimens were fixed in 2.5% phosphate buffered glutaraldehyde, washed in the same buffer, and then postfixed in 1% OsO₄. After dehydration in increasing concentrations of ethanol, the specimens were embedded in araldite (CY 212). Semithin sections were stained with toluidine blue. Ultrathin sections were contrasted with uranylacetate and lead citrate and examined under a Zeiss 952 electron microscope.

Genetic analysis
Microsatellite marker analysis
Samples of venous blood were obtained from patients and relatives after informed consent. The three families affected by GAN were genotyped with polymorphic markers present in the GAN region on chromosome 16q24.1 (LC8, LC6, LC5, D16S3098, LC1, LC3, LC2, and D16S505). Forward primers were labelled with 6-Fam, Hex, or Ned fluorochromes (Applied Biosystems Inc, Foster City, California, USA). Polymerase chain reactions (PCRs) were performed in a final volume of 10 μl containing 20 ng of genomic DNA, 1mM dNTP, 1× NBL buffer, 0.50 μM of each primer, and 0.1 IU of Taq polymerase. The fluorescent PCR products were separated and detected on 4.25% acrylamide gels with an ABI 377 DNA sequencer, and alleles were assigned with the Genotyper software (v2.5; Applied Biosystems).

Mutation analysis
The 11 exons of the GAN gene were screened for mutations at the genomic level. The sequence of the primers has been published (http://genetics.nature.com/supplementary info/). PCR products were analysed by single strand conformational polymorphism (SSCP) on PlusOne precast acrylamide gels (Pharmacia, Uppsala, Sweden) with a Genephor electrophoresis device (Pharmacia) at three different running temperatures (10°C, 15°C, and 20°C), and silver stained. Electrophoretic variants were sequenced from both the forward and reverse strands.

RESULTS

Clinical features
Table 1 outlines the clinical features of the six patients investigated. All patients had a similar clinical presentation, compatible with a degenerative disease. They were born after an uneventful pregnancy and delivery. Psychomotor development was normal in these patients. Onset of disease varied from 3.5 to 4.5 years of age. Distal limb weakness was the initial complaint in all patients. In addition, truncal ataxia contributing to unsteady gait was noticed at the beginning of the disease in two patients (patients 3 and 6). Cerebellar abnormalities became apparent within a few years in other patients. Mental deterioration was noted to start before the age of 10 years. All patients showed a progressive clinical course. Patients 1, 4, 5, and 6 became wheelchair bound around 9–10 years of age. None of the patients had seizures or seizure-like episodes.

Physical examination
All patients were of short stature, being under the third centile. They had pale and curled hair, known as frizzly hair, and dry skin. A peculiar facial appearance characterised by

facial diplegia, ptosis, and a prominent high forehead was present. Two female patients (patients 4 and 6) had early breast development consistent with pubertal precocious. Boys showed no signs of early pubertal development. Scoliosis was present in five patients (table 1). Other skeletal deformities consisting of various combinations of pectus carinatum, genu–valgus, pes equino–valgus, and pes planus were also noted in these five patients.

Neurological examination
All patients had mild mental retardation except for the youngest one (patient 3). Optic fundus examinations were normal. Five patients had slight facial weakness characterised by flattening of the nasolabial sulci and inability to raise the eyebrow. Ptosis was present in four patients. Most patients showed generalised hypotonia, which clearly predominated in the distal lower limbs. Pronounced distal muscle weakness and atrophy were present in the lower limbs. The power of the distal limb muscles ranged between 2 of 5 and 3+ of 5. Ankle jerks were absent. Weakness was less pronounced in the upper extremities. Patients 4, 5, and 6 presented moderate to severe upper limb weakness, with tenar–hypotenar atrophy of the hands, whereas milder muscle weakness with normal or hyperactive deep tendon reflexes was present in the other patients. Mild impairment of pain and light touch sensations was observed. All patients had truncal ataxia, nystagmus, dysmetria, and dysarthria. Plantar responses were extensor. There was no sign of spasticity.

Electroencephalography
Bilateral synchronous sharp wave discharges, most prominent in the left anterior regions, were seen in the EEG of patient 1. The EEG of patient 4 showed spike-slow wave discharges in central areas. Sharp wave and sharp-slow wave discharges in the right centroparietal and parietooccipital regions with spreading to homologous regions of the left hemisphere were seen in the EEG of patient 5. The EEGs of patients 2, 3, and 6 were normal.

Electromyography
The EMGs of patients 1, 2, 5, and 6 were compatible with sensory motor neuropathy of axonal type. Sensory action potentials were absent or significantly decreased. Motor nerve conduction velocity (MNCV) was slowed to the demyelinating range in the lower limbs of patients 2 and 5 (table 1). Patient 1 had normal MNCV values in the lower limbs. MNCV was normal or mildly slowed in the upper limbs of all patients. An EMG of the heterozygous carrier in family 1 (individual (II)-14) at age 41 years showed bilateral mild to moderately reduced compound nerve action potential amplitudes of the sural nerves, which was compatible with mild axonal neuropathy (left sural sensory nerve action potential, 2.1 μV; right sural sensory nerve action potential, 2.4 μV; normal value, > 5 μV). MNCV was normal in this individual. The heterozygous carrier in family 3 (individual (I)-1) had a normal EMG.

Magnetic resonance imaging
Table 2 outlines the MRI findings in four patients. MRI scans were available for patients 1, 2, 5, and 6 at ages 11, 10, 5, and 16 years, respectively. The MRI scans showed diffusely increased T2 and decreased T1 signal intensities in the anterior and posterior periventricular and cerebellar white matter of patients 1, 2, and 6, but minimal hyperintensity in the cerebellar white matter and no change in the periventricular white matter of patient 5 (figs 2 and 3). Subcortical white matter was spared in these patients except for patient 1, who had slight involvement of subcortical U fibres. The
posterior crus of the internal capsule showed increased signal intensity on T2 weighted images in cranial MRI scans of patients 1, 2, and 6. Increased T2 weighted signal intensities were present in the posteromedial thalami of patient 6. Atrophy of the cerebellum and cervical spinal cord and increased T2 weighted signal intensity of the brainstem were evident in patients 1 and 6. Two patients received contrast material, and contrast enhancement in parietal white matter and cerebellum was seen in the MRI scans of patients 2 and 6, respectively. The cavum septi pellucidi et vergae abnormality was present in all patients. The corpus callosum and basal ganglia were normal in all of the patients.

**Light microscopy**

Light microscopy of the sural nerve from patient 1 showed a moderate reduction in the number of myelinated fibres (fig 4). Several fibres with distended axons (up to 20–25 μm in diameter) and thin myelin sheaths were noted among normal appearing myelinated fibres.

**Electron microscopy**

**Nerve biopsy**

Electron microscopy examination of the sural nerve from patient 1 showed both normal and giant sized axons. The axoplasm of axonal swellings was completely filled with tightly packed neurofilaments (fig 5A). Myelin sheets surrounding the swollen axons were abnormally thin.

**Skin biopsy**

The cytoplasm of fibroblasts from patients 2 and 5 showed an accumulation of intermediate filaments forming whorls (fig 5B).

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**Table 1 Clinical data of the patients**

<table>
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<tr>
<th>Patients</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<td>4.5 y</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
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<td>Yes</td>
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<td>ND</td>
<td>43 m/sec</td>
<td>37.6 m/sec</td>
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<td>Sharp wave</td>
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<td>Normal</td>
<td>Spike-slow wave</td>
<td>Sharp wave</td>
<td>Sharp-slow wave</td>
</tr>
<tr>
<td>Motor capacity</td>
<td>WCB</td>
<td>Walking with aid</td>
<td>Walking</td>
<td>WCB</td>
<td>WCB</td>
<td>WCB</td>
</tr>
</tbody>
</table>

EEG, electroencephalography; F, female; M, male; MNCV, motor nerve conduction velocity; ND, not done; WCB, wheel chair bound; y, years; +, mild; ++, moderate.

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**Figure 2** (A–C) T2 weighted axial magnetic resonance imaging (MRI) of the brain in patients 1, 2, and 6, respectively, showing increased signal intensity in the anterior and posterior periventricular white matter (arrows). (D) T2 weighted axial MRI of the brain in patient 5 showing no signal change in the periventricular white matter.

**Figure 3** (A–C) T2 weighted coronal magnetic resonance imaging (MRI) of the brain in patients 1, 2, and 6, respectively, showing significantly increased signal intensity in the periventricular and cerebellar white matter (arrows). (D) T2 weighted coronal MRI of the brain in patient 5 showing minimally increased signal intensity in the cerebellum (arrows).
Other laboratory investigations
The index patient of family 1 (patient 1) and the patients from families 2 and 3 had normal blood glucose, renal and liver function, vitamin B12, and arylsulfatase enzyme activity. Vitamin B12, vitamin E, thyroid hormones, and the blood glucose concentration were also normal in the heterozygous carrier from family 1 (individual (II)-14), who had subclinical neuropathy.

Genetics
Haplotype analysis
All patients from the three consanguineous families were homozygous for at least seven consecutive markers encompassing the GAN locus, and the healthy siblings had different genotypes (fig 1). In addition, all six affected members from family 1 were homozygous for the same haplotype. The results confirm linkage to 16q24.1 for all three families.

The patients in families 2 and 3, originating from the same region of Turkey, shared a common allele for six of the seven closely linked polymorphic markers tested. However, alleles at marker D16S3098, which is located immediately distal to GAN, differed by two CA repeats between the two families (fig 1).

Mutation analysis
Individuals (III)-1 and (III)-7 in family 1 were studied by SSCP for the 11 exons of the GAN gene. The electrophoretic variants were sequenced, allowing identification of the same homozygous mutation for each branch of the family: a C to T transition at position 877 on the cDNA, resulting in the occurrence of a stop codon at amino acid position 293 (R293X) (fig 6). We identified a novel homozygous mutation in the affected individuals in families 2 and 3: a G to T transversion at position 1502+1, which affects the donor splice site of exon 9 (fig 6). The 1502+1G>T mutation was not identified in the 100 chromosomes from Turkish control subjects by direct sequencing.

DISCUSSION
Clinical features
All patients showed a homogeneous clinical picture, mainly characterised by the involvement of neuroectodermal systems, including peripheral neuropathy, CNS features, and frizzy hair. Peripheral neuropathy dominated the clinical picture at the beginning of the disease. Distal limb weakness caused by peripheral neuropathy was the presenting feature. Progressive sensory motor neuropathy was found to be primarily axonal. Secondary demyelination may also be associated with axonal degeneration in GAN.25 26

Table 2 Magnetic resonance imaging findings in the patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>White matter abnormality</th>
<th>Brainstem involvement</th>
<th>Cerebellar atrophy</th>
<th>Posterior crus of the internal capsule</th>
<th>Thalamic involvement</th>
<th>Cavum septi pellucidi et vergae</th>
<th>Basal ganglia abnormality</th>
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<td>1</td>
<td>Anterior–posterior PV, C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>2</td>
<td>Anterior–posterior PV, C</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>Minimal</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>Anterior–posterior PV, C</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

C, cerebellar; PV, periventricular.

Figure 4  Semithin section of the sural nerve from patient 1 showing several giant axons with thin myelin sheath (original magnification, ×1428).

Figure 5  (A) Electron microscopy of the sural nerve from patient 1 showing a giant axon filled with masses of neurofilaments (original magnification, ×152 500). (B) Accumulation of whorly intermediate filaments in fibroblasts from patient 5 (original magnification, ×77 500).
moderate to severely decreased motor nerve conduction velocities, presumably as a result of demyelination, in two of the four patients studied by EMG.

A generalised involvement of CNS structures such as the cerebral cortex, cerebellum, brainstem, and pyramidal tracts was shown in postmortem studies of patients with GAN.27 28 Cerebellar dysfunction was the most common CNS manifestation in our patients. It became symptomatic earlier than the other CNS symptoms. Cerebellar ataxia accompanied distal limb weakness at initial presentation in two patients. Cranial nerve impairment was restricted to facial and oculomotor nerves, causing facial weakness and ptosis, respectively. However, a more diffuse dysfunction of the brainstem, with involvement of multiple cranial nerves, was also described in patients with GAN.29 30 Mental deterioration started in a later phase of the clinical course and progressed slowly.

Pubertal precocious is an unusual and rare clinical feature of GAN. Two female patients had early breast development according to their chronological age. It has been suggested that this feature is caused by CNS involvement.31 32 However, no sign of pubertal precocious was present in the male patients at similar ages.

Our patients had a severe clinical phenotype, which presented in a stereotypical fashion as outlined above, in contrast to some unusual presentations of GAN in the literature. Predominant expression of CNS involvement, including epileptic seizures and mental deterioration, was seen in a patient with GAN.1 24 However, no sign of pubertal precocious was present in the male patients at similar ages.

EEG features
Electrophysiological evidence of CNS involvement was present in three patients. Disorganised background activity with focal spikes, spike-slow wave discharges, and paroxysmal slow wave activity were reported in GAN.10 33 34 None of our patients had seizures, but focal or generalised EEG abnormalities characterised by sharp wave paroxysms, sharp-slow wave, and spike-slow wave discharges were seen in three patients (table 1). These discharges showed no particular distribution, originating from centroparietal, anterior, or posterior regions, sometimes with secondary generalisation.

MRI features
Neuroimaging findings described in GAN include widespread white matter demyelination and atrophy of the cerebellum, brainstem, spinal cord, and corpus callosum.34 35 36 White matter abnormality was the most common MRI finding in our patients. In patients 1, 2, and 6, diffuse white matter lesions were present in the anterior and posterior periventricular regions and cerebellum, extending into the subcortical white matter. However, cranial MRI of patient 5 showed limited involvement of the cerebellar white matter, perhaps because MRI was carried out at an earlier age in this patient. Progression of white matter lesions has been shown in patients with GAN.35 White matter involvement of GAN does not establish a specific pattern. Fronto-parietal and periventricular white matter are affected more prominently in some patients with GAN, even sometimes sparing the cerebellum.33 37 In contrast, more pronounced involvement of the cerebellar and occipital white matter compared with the frontal regions was also seen in GAN.33 37 Demyelination with gliotic changes, few giant axons, and numerous astrocytic processes and Rosenthal fibres were reported microscopically in the white matter lesions of patients with GAN.35 In accordance with the histopathological features, recently, cerebral proton magnetic resonance spectroscopy of a patient with GAN showed findings of demyelination in the white matter, and increased amounts of choline containing compounds and myo-inositol.37

The cavum septi pellucidi and vergae abnormalities were detected in the MRI of all patients, and have also been noted in other patients with GAN.36 Cavum septi pellucidi is considered to be a significant marker of aberrant brain development and is rarely seen in normal individuals (2.4%), whereas the cavum vergae deformity occurs with the same frequency in both normal and retarded populations.34 Cavum septi pellucidi results from the failure of two primordial
leaflets of the septum to fuse. Although its clinical importance is not known, an increased frequency of cavum septi pellucidum was found in another white matter disorder—megalencephaly and leukodystrophy with a mild clinical course. 19

GAN mutations

In agreement with the known consanguinity of the parents, all affected children were homozygous for the mutation. We have already reported the R293X mutation found in family 1 in another Turkish family, where the patient was a compound heterozygote together with a C570Y mutation. 12 The R293X mutation is predicted to truncate a part of the first kch domain and five other kch domains. In an expression study, the R293X mutation was shown to cause loss of interaction between gigaxonin and MAP1B-LC. 21 Because this mutation alters a CG dinucleotide, which are known to be mutational hot spots by transition of methylated C to T, the nucleotide change in both families may represent independent recurrent mutational events. However, patients in both families shared the same homozygous alleles for markers LC5 and LC6 (data not shown). According to the complete sequence of the gene from the human genome working draft, LC5 is an intragenic marker located between exons 5 and 6. Therefore, it is equally possible that the multiple occurrence of the R293X mutation in Turkey is the consequence of a common founder event.

Patients from families 2 and 3 share the same mutation, which is a splice mutation of exon 9 (1502+1G>T). Skipping of this exon is predicted to result in an in frame transcript missing most of the sequence encoding the fifth kch repeat. The two families originate from nearby towns in southeastern Turkey and share a common haplotype over three consecutive intermarkers (LC1, LC3, and LC2), but are divergent at the intermediate marker D16S3098. This divergence may be the consequence of mutational instability at the D16S3098 marker locus, or less probably may reflect the occurrence of an ancestral recombination immediately distal to the GAN gene. The results presented here indicate that an initial screen for mutations in exons 5 and 9 of Turkish patients with GAN may be an efficient strategy.

Both mutations are predicted to have serious affects on the structure of gigaxonin. The associated phenotypes of the two mutations in the homozygous state were similar, except for the milder involvement of the white matter in one of the patients with the 1502+1G>T mutation. Similar widespread white matter changes with brainstem and cerebellar atrophy were also seen in the cranial MRI of a patient with GAN who had a homozygous A to G transition in the splice donor site of intron 3, resulting in abnormally spliced gigaxonin. 17 More cases are needed to make a comparison between the mutation type and CNS involvement in GAN.

Recently, using EMG, Kuhnlenbämmer et al found subclinical signs of peripheral neuropathy in a heterozygous parent carrying a nonsense mutation (R201X), whereas another parent with a missense mutation (I423T) had a normal EMG. 13 In our study, EMG revealed mild axonal neuropathy in the carrier of the R293X mutation but not in the carrier of 1502+1G>T. This result may relate to the more severe gigaxonin truncation caused by the R293X mutation compared with the 1502+1G>T mutation. Similarly, the truncation of a part of the BTB domain and six kch domains by the R201X mutation described by Kuhnlenbämmer and colleagues 15 was sufficient to cause subclinical neuropathy in the heterozygous parent.

In conclusion, we screened the GAN gene for mutations in three Turkish families concordant for linkage to the GAN locus and identified two null mutations: the R293X mutation, previously reported in a small Turkish family, and the 1502+1G>T mutation. Both mutations were associated with white matter abnormalities in cranial MRI. No major intrafamilial or interfamilial clinical variability was present among the patients carrying these two mutations. We also found subclinical neuropathy in a heterozygous carrier of the R293X mutation. These mutations leading to the truncation of gigaxonin caused a severe clinical phenotype in our patients. However, the association of severe mutations with milder phenotypes suggests the lack of a correlation between the genotype and the clinical phenotype in GAN. 16 Further studies on the expression and function of gigaxonin may provide a better understanding of the pathophysiological mechanism of this disease.

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