## ELECTRONIC LETTER

# A novel Gypsy founder mutation, p.Arg1109X in the CMT4C gene, causes variable peripheral neuropathy phenotypes

R Gooding*, J Colomer*, R King, D Angelicheva, L Marns, Y Parman, D Chandler, J Bertranpetit, L Kalaydjieva

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**Background:** Linkage, haplotype and sequencing analysis in a large Spanish Gypsy kindred with multiple members affected by autosomal recessive peripheral neuropathy led to the identification of a novel mutation, p.Arg1109X, in the CMT4C gene. The screening of further unrelated patients, and of a panel of ethnically matched controls, showed that p.Arg1109X is an ancestral mutation which occurs in Gypsy populations across Europe and is the most common cause of autosomal recessive Charcot–Marie–Tooth disease in Spanish Gypsies.

**Objective:** To report the identification of a novel Gypsy founder mutation causing autosomal recessive CMT4C disease in a sample of homozygous affected individuals.

**Results:** The mutation was associated with a surprisingly broad spectrum of neuropathy phenotypes, with variation in the age at onset, rate of progression, severity of muscle and sensory involvement, the presence of scoliosis, and cranial nerve involvement.

**Conclusions:** Ascertainment and further studies of CMT4C patients in this population will provide a unique opportunity for characterising the full range of clinical manifestations of the disease in a genetically homogeneous sample.

Autosomal recessive forms, caused by private founder mutations, account for most cases of demyelinating Charcot-Marie-Tooth (CMT) disease among European Gypsies and represent a considerable health burden and cause of disability in many Gypsy communities.[1 2] Three novel conditions, associated with hypo/demyelinating peripheral neuropathy, have so far been identified and characterised in this population: hereditary motor and sensory neuropathy Lom, HMSNL,[3–5] hereditary motor and sensory neuropathy Russe, HMSNR,[6–8] and congenital cataracts facial dysmorphism neuropathy (CCFDN) syndrome,[9–11] of which HMSNL is the most common and widespread.[2] While ocular abnormalities distinguish the CCFDN syndrome,[10 12] the differential diagnosis between HMSNL and HMSNR may be difficult in the early stages of the disease.[13] Genetic analysis is thus a valuable diagnostic tool, especially in extended kindreds with independent segregation of more than one form of peripheral neuropathy.[6 9] Our experience with diagnostic investigations of Gypsy patients from many European countries suggests that the three known conditions do not explain all cases of autosomal recessive CMT disease in this population, and that additional forms remain to be characterised.

Here we report the identification of a novel Gypsy founder mutation causing autosomal recessive CMT4C disease, a premature termination codon in the SH3TC2 (KIAA 1985) gene,[14] and present data on its frequency and geographical distribution in European Gypsies. The variable clinical manifestations observed in our homozygous patients point to the complexity of genotype–phenotype correlations in CMT4C disease and suggest that the ancestral Gypsy mutation provides a unique opportunity for characterising the broad phenotypic spectrum of CMT4C in a genetically homogeneous group of affected individuals.

## METHODS

### Subjects

The study included 19 affected individuals from 11 unrelated Gypsy families with autosomal recessive demyelinating CMT disease, residing in different European countries. Informed consent was obtained from all subjects. The study complies with the ethics guidelines of the institutions involved.

Of the 12 patients found to be homozygous for the newly identified CMT4C mutation, eight belonged to a large Spanish Gypsy kindred (fig 1A; patient IDs from A to E in table 1) scattered throughout Spain and recruited in the course of several years for participation in the study. The affected pedigree members were related to the proband (IV-10 in family A, fig 1A) on both sides, through the maternal and the paternal grandfather. Two additional cases came from unrelated Gypsy families from other parts of Spain (detailed clinical information available on Sp3–3, table 1). Another Gypsy family with two affected children (P14–3 and P14–4 in table 1) was referred from Turkey but was originally from Albania. None of the parental couples reported consanguinity.

The homozygous patients presented with diverse peripheral neuropathy phenotypes, summarised in table 1 (cases P14–3 and P14–4 have also been described by Parman[15]). The age at onset ranged from the first months of life to the fourth decade. Some patients never walked, while others were still ambulant in their fifth decade. Muscle involvement, especially proximal, varied widely. The extent of sensory loss was also variable, with sensory ataxia being the most disabling symptoms in some patients and totally absent in others. Only three of 12 affected subjects had scoliosis. In a subset of individuals (branches C and D of the large Spanish kindred and the P14 family from Turkey) the phenotype was characterised by cranial nerve involvement with deafness, making it difficult to differentiate on clinical grounds from HMSNL.[4] Electrophysiological data in all cases were compatible with a primarily demyelinating neuropathy with

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**Abbreviations:** CCFDN, congenital cataracts facial dysmorphism neuropathy syndrome; CMT, Charcot–Marie–Tooth disease; HMSNL, hereditary motor and sensory neuropathy Lom; HMSNR, hereditary motor and sensory neuropathy Russe
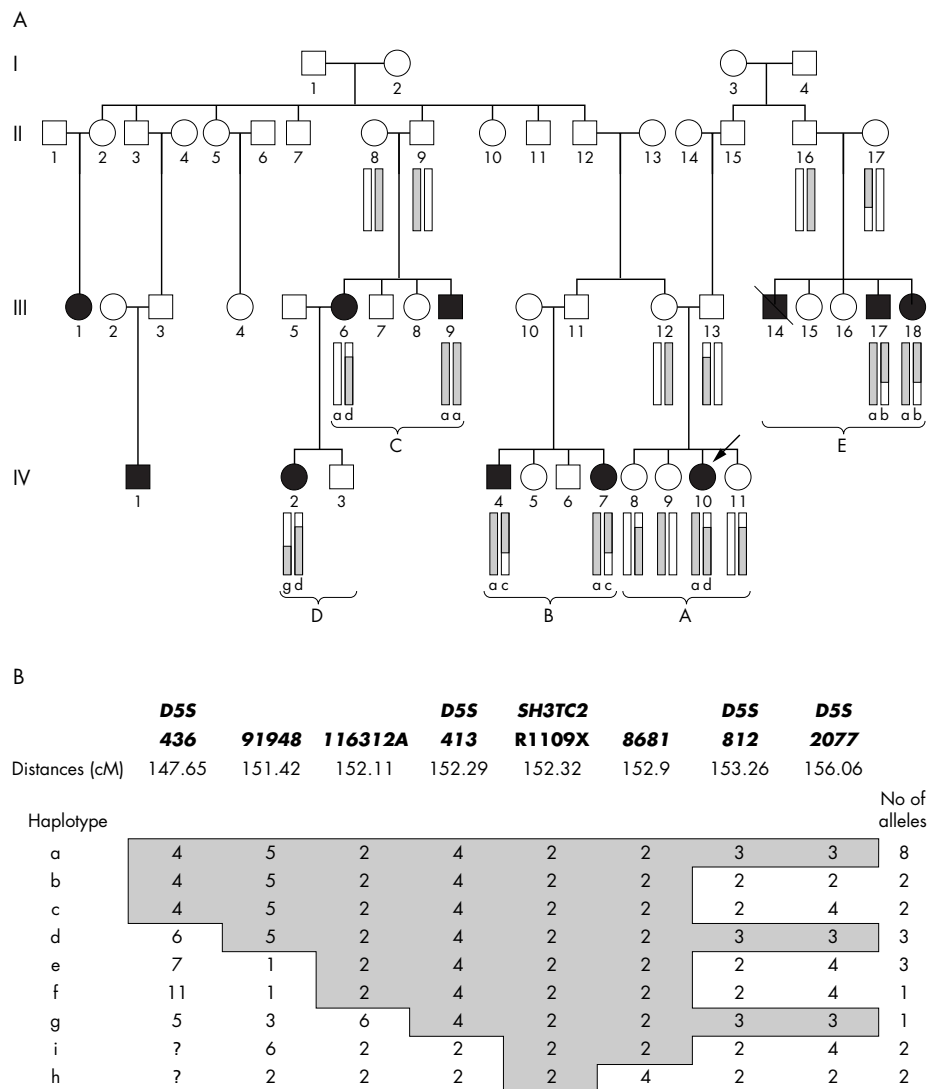
A

B

| | D5S 436 | 91948 | 116312A | D5S 413 | SH3TC2 R1109X | 8681 | D5S 812 | D5S 2077 | No of alleles |
|---|---|---|---|---|---|---|---|---|---|
| Distances (cM) | 147.65 | 151.42 | 152.11 | 152.29 | 152.32 | 152.9 | 153.26 | 156.06 | |
| Haplotype | | | | | | | | | |
| a | 4 | 5 | 2 | 4 | 2 | 2 | 3 | 3 | 8 |
| b | 4 | 5 | 2 | 4 | 2 | 2 | 2 | 2 | 2 |
| c | 4 | 5 | 2 | 4 | 2 | 2 | 2 | 4 | 2 |
| d | 6 | 5 | 2 | 4 | 2 | 2 | 3 | 3 | 3 |
| e | 7 | 1 | 2 | 4 | 2 | 2 | 2 | 4 | 3 |
| f | 11 | 1 | 2 | 4 | 2 | 2 | 2 | 4 | 1 |
| g | 5 | 3 | 6 | 4 | 2 | 2 | 3 | 3 | 1 |
| i | ? | 6 | 2 | 2 | 2 | 2 | 2 | 4 | 2 |
| h | ? | 2 | 2 | 2 | 2 | 4 | 2 | 2 | 2 |

**Figure 1** (A) Pedigree structure of the large Spanish Gypsy kindred, recruited from different parts of Spain in the course of six years, where the novel founder mutation p.Arg1109X in the *SH3TC2* gene was identified. Capital letters refer to the designations of the nuclear families within the kindred, as used in the description of the patients' clinical phenotypes in table 1. The bars shown under the subjects included in the genetic analysis designate the five CMT4C haplotypes segregating in the kindred, with the shaded area representing the conserved ancestral haplotype. (B) Chromosomal haplotypes, formed by seven microsatellite markers around the CMT4C locus, spanning an overall genetic distance of 8.4 cM. In all, nine haplotypes were observed in the 24 disease chromosomes, of which five (shown in panel A) segregated in the original large affected kindred and four were found in the additional affected subjects from Spain (haplotypes e and f) and Turkey (i and h). Intermarker genetic distances were taken from the integrated genetic map available at the website of the Queensland Institute for Medical Research (QIMR) or, for markers identified in the Senderek *et al* study,[14] interpolated using the QIMR approach. Assuming equidistant positions of the recombinations relative to the flanking markers, the conserved ancestral haplotype had a total length of ~180 kb, spanning a genetic distance of ~0.3 cM.

secondary axonal involvement. Nerve conduction velocities for the median nerve ranged between 13 and 30 m/s in our sample, similar to those reported for other CMT4C patients.[14 16]

Neuropathological investigations were done on sural nerve biopsies from patients A IV-10 and P14–3. In P14, demyelination with multiple onion bulbs and severe axonal loss were observed.[15] The biopsy of A IV-10 was obtained at age 12 years during corrective orthopaedic surgery. Light microscopy showed a moderately severe loss of myelinated fibres; those remaining were predominantly of small diameter (fig 2A). There were some signs of recent axonal degeneration and small numbers of regenerative clusters. Some demyelinated axons were present and many of the myelin sheaths were inappropriately thin for axon diameter. There were some onion bulb formations. Extensive collagen deposition and

some subperineurial oedema was observed. Electron microscopy (fig 2B) showed axons surrounded by numerous layers of redundant basal laminae, known as "basal laminal onion bulbs." Myelinated, thinly myelinated, or demyelinated axons were all found in the centre of basal laminal onion bulbs. Some Schwann cells associated with myelinated fibres had unusual thin processes protruding into the endoneurium. The Schwann cells associated with unmyelinated axons were also abnormal, possessing very thin processes that were reduced to apposed cell membranes.

## Molecular analyses
Genomic DNA was extracted from blood samples using standard techniques.

The p.Arg148X mutation in *NDRG1* (causing HMSNL) and linkage to the HMSNR locus were analysed as described.[5 6]

**Table 1** A summary of the clinical manifestations in patients homozygous for the *SH3TC2* p.Arg1109X mutation

| Patient ID | Age at walking (m) | Age at diagn (y) | Curr age (y) | Muscle weakness and atrophy* | | | | | Sensory loss* | | | Cranial nerve involve | Degree of disability* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Lower limbs distal | Lower limbs prox | Upper limbs distal | Foot deformity* | Scoliosis* | Touch | Vibr | Proprio | | |
| A IV-10 | 13 | 7 | 20 | +++ | ++ | + | +++ | Mod | Yes | Yes | No | No | Unstable ("clumsy") gait |
| B IV-4 | 13 | 6 | 28 | +++ | + | + | + | No | Yes | Yes | Yes | No | "Clumsy" gait |
| B IV-7 | 14 | 6 | 23 | +++ | + | + | (+/−) | No | Yes | Yes | Yes | No | "Clumsy" gait |
| C III-6 | 14 | 37 | 47 | +++ | +++ | + | ++ | No | Yes | Yes | Yes | Yes | Stands with support |
| D III-9 | 12 | 26 | 43 | +++ | +++ | + | (+/−) | No | Yes | Yes | Yes | Yes | Walks with support |
| D IV-2 | 13 | 16 | 25 | ++ | + | + | + | No | Yes | Mild | Mild | Yes | Ambulant |
| E III-14 | Never walked | Infancy | Died at 22 | U/K | U/K | U/K | U/K | U/K | U/K | U/K | U/K | U/K | Never walked, wheel chair bound |
| E III-17 | 30 | Infancy | 14 | +++ | +++ | +++ | +++ | Severe | Yes | Yes | Yes | No | Never walked, wheel chair bound |
| E III-18 | 7 | 7 | 8 | + | + | + | + | No | Yes | Yes | Yes | No | Walks with support; severe ataxia |
| Sp 3-3 | 26 | 4 | 9 | +++ | + | ++ | + | Yes | Yes | Yes | Yes | No | "Clumsy" gait, ataxia |
| P14-3 | 12 | 29 | 41 | +++ | + | ++ | Pes cavus, hammer toes | No | Yes | Yes | Yes | Yes | "Clumsy" gait |
| P14-4 | 12 | 37 | 46 | +++ | + | ++ | | No | Yes | Yes | Yes | Yes | "Clumsy" gait |

*The assessments were obtained at recent examinations and refer to the current age of the patients.
Curr, current; diagn, diagnosis; ID, identification number; involve, involvement; m, months; mod, moderate; Proprio, proprioception; prox, proximal; U/K, unknown; Vibr, vibration; y, years.

A 10 cM genome-wide scan was conducted in three branches of the large Spanish kindred, using the Linkage Mapping Set v2.5-MD10 (Applied Biosystems, Foster City, California, USA). Fine mapping of the CMT4C region was carried out in the entire kindred by genotyping additional microsatellite markers D5S413, D5S812, and D5S2077 (selected from public genome databases), and 91948, 116312A, and 8681, reported by Senderek *et al*.[14] These markers—spanning a genetic distance of 8.4 cM—were also used in the haplotype analysis of all mutant chromosomes identified throughout the study. Electrophoretic length separation of the PCR products was done on an ABI 377 DNA analyser. The data were analysed with the Genotyper software (Applied Biosystems). Linkage analysis was done using GENEHUNTER version 2.1, under an autosomal recessive model with complete penetrance, gene frequency set at 0.01, and equal allele frequencies. The genetic distances between markers included in the CMT4C haplotypes were estimated using the integrated genetic map (produced by David Duffy at the Queensland Institute for Medical Research), which contains interpolated genetic map positions estimated through locally weighted linear regression from the physical map and the published DeCODE and Marshfield maps.

Sequencing of all coding regions and flanking introns of the *SH3TC2* gene was carried out on genomic DNA from members of the large kindred, the affected individuals representing the different phenotypes, and an obligate carrier parent and an unaffected sibling presumed to be a non-carrier based on haplotype analysis. We used BigDye chemistry (Applied Biosystems) and the published primers.[14] The products were run on an ABI 377 DNA analyser and analysed with the Sequence Navigator software (Applied Biosystems). The sequence information was compared between the samples included in the analysis, as well as to the NCBI *SH3TC2* reference sequences NM_024577 (*H sapiens* mRNA), NC_00005 (chromosome 5 genomic sequence, nucleotide positions 148,364,377–148,422,880), and NP_078853 (*H sapiens* protein).

A PCR based RFLP assay (*Dde*I site created by the mutation), with fluorescent labelling of the forward primer and detection of the restriction fragments on a 377 DNA analyser (fig 3B), was used for mutation screening.

The screening was carried out on DNA samples from 11 additional Gypsy subjects from different parts of Europe, affected by unidentified autosomal recessive forms of demyelinating CMT disease.

The frequency of the mutation in the general Gypsy population and its distribution in different subisolates were analysed in a panel of 522 anonymous unrelated controls: 367 subjects from different Gypsy groups in Bulgaria[2] and 155 from Spain (79 from the area of Madrid and 76 from Barcelona).

## RESULTS
### Linkage analysis
After excluding the p.Arg148X HMSNL mutation and linkage to the HMSNR locus, we conducted a genome scan on branches A, C, and D of the large Spanish Gypsy kindred, which were available at the time. This analysis excluded large parts of the genome and pointed to three regions as the best candidates for further investigations: 5q32-q33 (multipoint lod score 1.74 at D5S410), 7q36 (1.57 at D7S636), and 10p14 (1.5 at D10S547). The 5q locus was supported further by the analysis of the newly recruited branches of the kindred (B and E) and the fine mapping results, with the combined maximum multipoint lod score for the entire kindred reaching 3.08 at marker D5S413.
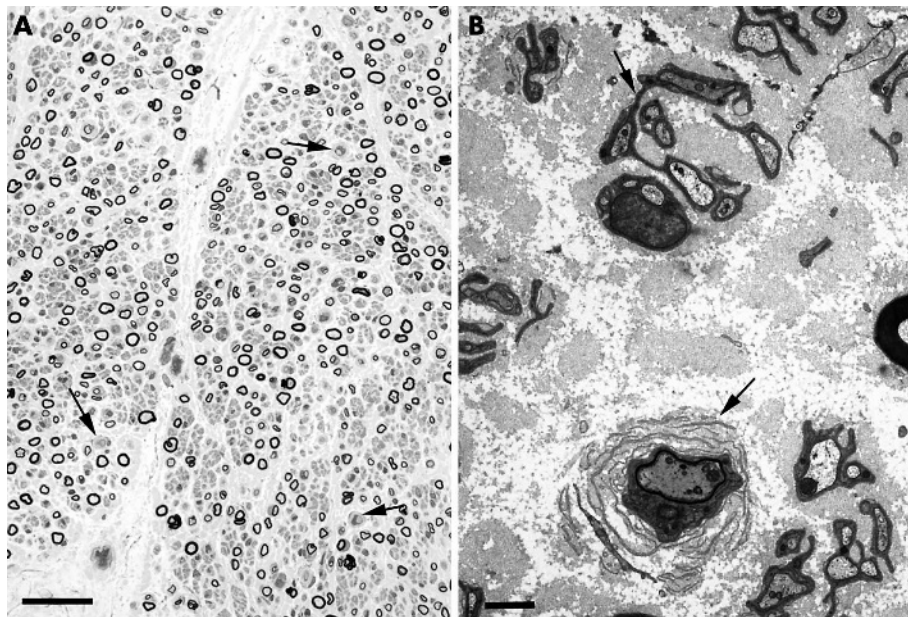
**Figure 2** Neuropathological findings in a sural nerve biopsy from the index patient. (A) Resin section showed a reduced population of myelinated fibres. Many fibres have myelin sheaths that are inappropriately thin for the axon diameter. There are occasional demyelinated axons (arrows). Bar = 50 μm. (B) Electron microscopy identified occasional fibres encircled by reduplicated basal laminae (basal laminal onion bulbs) (lower arrow). The Schwann cells of unmyelinated axons often had very thin processes and connecting links between axons (upper arrow). Bar = 2 μm.

The linked 5q region contains the CMT4C locus, with our highest lod score observed at a marker very close to the recently identified gene KIAA 1985 or the SH3 domain and tetratricopeptide repeats 2 gene (*SH3TC2*).[14] In search of the disease causing mutation, we sequenced all 18 exons and flanking intronic sequences of *SH3TC2* in members of the large affected kindred. The analysis identified a novel mutation, a C→T change in the last codon of exon 14 of *SH3TC2* (c.3325, g.52751, CGA→TGA) (fig 3A), predicted to result in a premature termination signal at amino acid position 1109, Arginine → Stop, p.Arg1109X. The mutation was present in the homozygous state in all three affected subjects, regardless of the clinical differences. Analysis of the entire large kindred showed perfect co-segregation with the peripheral neuropathy phenotype.

The pedigree structure, with many reportedly unrelated subjects contributing the same mutation, suggested a high carrier rate, particularly in the Spanish Gypsy population. To check whether it occurs in other Gypsy patients with autosomal recessive demyelinating CMT disease where the molecular cause has remained unidentified, we tested 11 additional affected subjects: six unrelated individuals from Spain, one each from Romania, Hungary and Ireland, and two siblings from Turkey. The p.Arg1109X mutation was indeed detected in four cases: two Spanish patients and the affected brothers in the Turkish family were homozygous for the mutation.

Haplotype analysis of p.Arg1109X chromosomes revealed a diversity of historical recombinations, with five different disease haplotypes segregating in the large affected kindred (fig 1, panels A and B). Only two of the 12 p.Arg1109X homozygotes were also homozygous for the polymorphic haplotype. The conserved ancestral segment spans a small genetic distance, with the two markers flanking the recombination breakpoints separated by ~0.6 cM (fig 1B).

The detection of p.Arg1109X in patients from Spain as well as Turkey, and the small size of the shared conserved haplotype, suggested an ancestral mutation whose origin predates the geographical dispersal of the Gypsies in Europe.[2] To determine its distribution and frequency, we screened a panel of 522 unrelated control subjects of Gypsy ethnicity, representing Gypsy populations with a different social and biological history.[2 17] Seven heterozygous individuals were identified, translating into an overall carrier rate of about 1 in 75. One of the carriers was from Bulgaria, while the remaining six were Spanish Gypsies (two from Madrid and four from Barcelona), where the frequency of p.Arg1109X heterozygotes can be estimated at around 1 in 26.

## DISCUSSION

In this study, we have identified the fourth founder mutation associated with demyelinating peripheral neuropathy in the European Gypsy population. The detection of p.Arg1109X in CMT patients and in population controls from distant parts of the continent suggests that the mutation was present in the early Gypsy population prior to its diaspora in Europe and the splits into endogamous subisolates which began around 700 years ago.[2 17] This is supported by the observed haplotype diversity, related to multiple historical recombinations and high gene frequencies—a common characteristic of ancestral Gypsy mutations,[2 18 19] which precludes the use of standard homozygosity mapping even in highly inbred pedigrees unless relying on intragenic or very closely linked polymorphisms. As with other founder mutations, p.Arg1109X was unevenly distributed in the genetically structured European Gypsy population.[2 19] Our current results suggest a relatively low frequency in the Eastern European Roma, which may reflect the random genetic drift or the underrepresentation of the relevant Gypsy groups in our population control panel. By contrast, the high proportion of affected families and the ~4% carrier rate in population controls from Spain point to CMT4C as the most common form of autosomal recessive demyelinating CMT disease among Spanish Gypsies. p.Arg1109X testing should thus become part of the diagnostic panel for CMT patients of Gypsy ethnicity, especially taking into account the diversity of clinical manifestations observed in our study and their close resemblance, in some cases, to the HMSNL phenotype.[4]

The recently identified CMT4 gene, *SH3TC2*, encodes a novel large protein of 1288 amino acids with two N-terminal
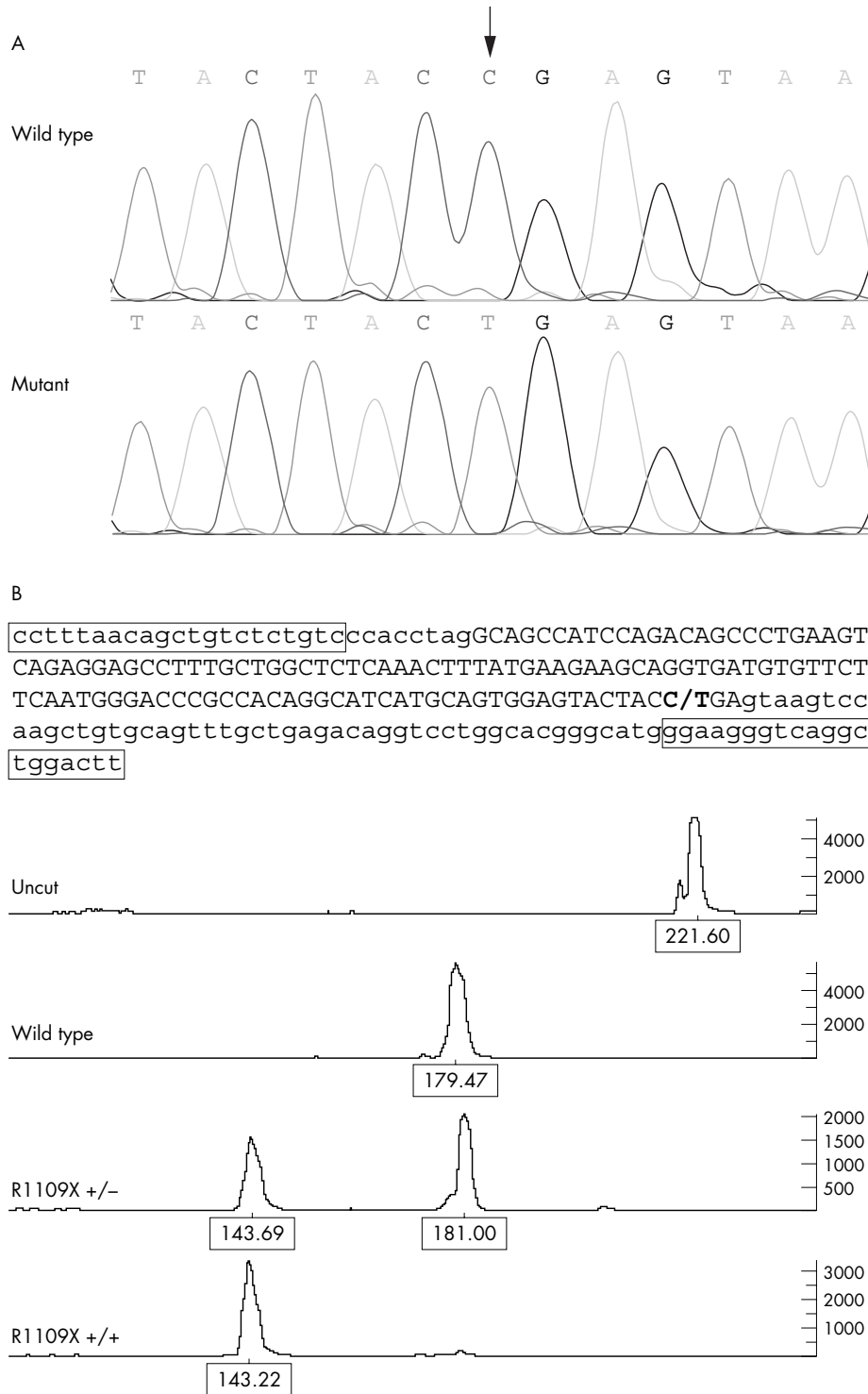
A

Wild type

T A C T A C C G A G T A A

Mutant

T A C T A C T G A G T A A

B

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cctttaacagctgtctctgtcccacctagGCAGCCATCCAGACAGCCCTGAAGT
CAGAGGAGCCTTTGCTGGCTCTCAAACTTTATGAAGAAGCAGGTGATGTGTTCT
TCAATGGGACCCGCCACAGGCATCATGCAGTGGAGTACTACC/TGAgtaagtcc
aagctgtgcagtttgctgagacaggtcctggcacgggcatgggaagggtcaggc
tggactt
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Uncut

221.60

Wild type

179.47

R1109X +/−

143.69

181.00

R1109X +/+

143.22

**Figure 3** (A) Sequencing electrophoregrams of exon 14 of *SH3TC2*, showing the C→T transition which affects the last codon of the exon (amino acid position 1109), changing it from Arginine to Stop (p.Arg1109X). (B) The *Dde*I restriction site created by the nucleotide substitution was used for the design of an RFLP based mutation screening test. The forward PCR primer (primers are boxed) was fluorescently labelled, and the digested PCR products were separated on an ABI 377 DNA analyser, producing the pattern shown in the lower part of the figure. The screening of a panel of 522 anonymous controls revealed carrier rates of 1:75 overall and 1:26 among Spanish Gypsies. PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

Src homology 3 domains and 10 tetratricopeptide repeat motifs.[14] While sequence homology to known protein domains suggests involvement in protein–protein interactions and a putative classification as a docking or adapter protein,[14] the cellular function of SH3TC2 and its unique role in the peripheral nervous system remain to be elucidated. The p.Arg1109X mutation belongs to the most common category of molecular defects observed by Senderek and colleagues[14] in *SH3TC2*, namely premature termination codons and frameshift mutations predicted to result in protein truncation. Its

localisation close to the C-terminal end of the protein (similar to the reported Y943X, R954X, P1114fsX1115, and Q1201X mutations) provides further support to the conclusion that the integrity of the longest *SH3TC2* product is crucial for normal peripheral nerve function.[14]

*SH3TC2* mutations appear to account for a significant proportion of patients with autosomal recessive forms of CMT disease: in the original study by Senderek *et al*, 15 of 55 families showed linkage to the CMT4C locus, and mutations in the gene were detected in two of 21 sporadic cases.[14] Outside the Gypsy population, CMT4C has a ubiquitous ethnic distribution, with many cases detected in Mediterranean countries and in a sample of Dutch families.[16 20 21] The gene has been localised by linkage analysis of two consanguineous Algerian families,[21] while fine mapping and positional cloning relied on an ethnically heterogeneous collection of patients and families.[14] The finding of multiple private defects confined to single families has made it difficult to draw conclusions on genotype–phenotype correlations.[14] The founder p.Arg1109X mutation thus provides an opportunity for understanding the clinical spectrum of CMT4C in a patient population sharing a single ancestral mutation and a less diverse genetic background. Our current data indicate that *SH3TC2* defects may be associated with a broad range of clinical manifestations which cannot be explained by allelic heterogeneity and point to the existence of modifying factors. Further delineation of phenotypic subtypes and research into the causes of clinical diversity may be possible with the recruitment of large numbers of subjects homozygous for p.Arg1109X which, based on our previous experience with other Mendelian disorders in the Gypsies, is an achievable goal. Given the proposed SH3TC2 function as a docking protein involved in multiple protein–protein interactions, the possibility of genetic variation in the protein partners contributing to the end point clinical phenotype merits further investigation.

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## ELECTRONIC DATABASE INFORMATION

Genetic data and references: http://www.ncbi.nlm.nih.gov, http://genome.ucsc.edu/

Integrated genetic map: http://www.qimr.edu.au/davidD

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