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Genetic characterization of Charcot-Marie-Tooth disease type 4C in a Gypsy population

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Abstract

Charcot-Marie Tooth (CMT) disease type 4 (CMT4) refers to autosomal recessive forms of hereditary motor and sensory neuropathy (HMSN). Three neuropathies have been identified in the European Roma (Gypsy) people: HMSN-Lom (MIM 601455), HMSN-Russe (MIM 605285) and the congenital cataracts facial dysmorphism neuropathy syndrome (CCFDN; MIM 604168) that can be clinically distinguished because of the ocular abnormalities. We have carried out genetic and mutation analyses in a series of 20 Gypsy families from Spain with a diagnosis of a demyelinating CMT disease compatible with an autosomal recessive pattern of inheritance. We have found the p.R148X mutation in the *NDRG1* gene responsible for the HSMN-Lom in four families and, possible linkage to the HSMN-Russe locus in three cases that share the haplotype with other European HSMN-Russe families. We have also studied the CMT4C locus because of the clinical similarities and we have demonstrated that the disease is mainly caused by mutations located on the *SH3TC2* gene responsible for CMT4C (10 out of 20 families). Patients from 9 out of 10 families carried the p.R1109X mutation in homozygous state, while in one family patients were compound heterozygous, p.R1109X/p.C737_P738delinsX. We have also observed that the p.R1109X mutation is associated with a conserved haplotype and therefore, these mutant chromosomes may have a common origin; p.R1109X mutation may be a private founder mutation for Gypsy population. The estimation of the allelic age revealed that the p.R1109X mutation probably arose about 200 years ago, likely as the consequence of a bottleneck.

Keywords Charcot-Marie-Tooth disease type 4, Gypsy population, Founder effect, *SH3TC2* gene

Introduction

Inherited peripheral neuropathies are traditionally classified according to the predominant pathogenic mechanism causing the disorder and the peripheral system involved in the process. Charcot-Marie-Tooth (CMT) disease is a motor and sensory hereditary neuropathy that affects approximately one in 2,500 people, and is among the most common inherited neurological disorders [1, 2]. The majority of patients have autosomal dominantly inherited neuropathies that are separable into demyelinating (CMT1) or axonal (CMT2) forms of CMT [3]. CMT1 patients have slow nerve conduction velocities (NCV) and features of demyelination on nerve biopsies whereas CMT2 patients have normal or near normal NCV and pathological evidence of axonal damage without demyelination [3, 4]. Some patients develop autosomal recessively inherited forms of CMT (CMT4), which are particularly severe and disable the patient in infancy. Both demyelinating and axonal forms of CMT4 exist.

The Roma (Gypsies) is a transnational people that arrived into Eastern Europe approximately 1,000 years ago. The 8-10 million Roma living in Europe are considered as a conglomerate of genetically isolated founder populations [5]. Since the publication of the hereditary motor and sensory neuropathy Lom (HMSN-Lom, CMT4D; MIM 601455), a form of CMT associated with deafness, in Gypsy families from the town of Lom in Bulgaria [6, 7], a number of rare autosomal recessive disorders have been reported in the Roma people [8]. Three of these disorders are peripheral neuropathies: 1) the above mentioned HMSN-Lom, caused by the founder mutation p.R148X in *NDRG1* gene located on chromosome 8q24 [9]; 2) the hereditary motor and sensory neuropathy Russe (HMSN-Russe; MIM 605285) [10, 11] mapped on chromosome 10q23

[12]; and the congenital cataracts facial dysmorphism neuropathy (CCFDN; MIM 604168) [13], for which a founder mutation, a C>T substitution in an intronic Alu element, has been reported in the *CTDP1* gene that is located on chromosome 18q23 [14, 15].

We have carried out genetic and mutation analyses in a series of 20 Gypsy families from Spain, 13 of them coming from the Land of Valencia and the rest from other Spanish regions. We have found the p.R148X mutation in four families and possible linkage to the HSMN-Russe locus in three families. Moreover, we have also demonstrated that the disease is mainly caused by mutations in the *SH3TC2* (*KIAA1985*) gene that underlies CMT4C what highlights the genetic heterogeneity and complexity associated with CMT disease.

Methods

Study subjects

Twenty non-related Gypsy families from Spain with a diagnosis of a demyelinating CMT disease were available for genetic analysis. The cohort was distributed as follows: 8 isolated cases and 12 families with autosomal recessive inheritance (categorised thus because more than one sib was affected and parents were normal by examination or history). Thirteen families reside in the Land of Valencia and the remaining lives in the regions of Madrid (3), Andalusia (3) and Extremadura (1). Information about the geographical origin of the proposita or their ancestors was not gathered.

The thirteen Valencian families were supervised at the Department of Neurology of the La Fe University Hospital in Valencia and complete clinical records from probands and from some secondary cases were available. Electrophysiological and pathological studies suggested that patients from most of non-typed families showed early onset demyelinating neuropathy, frequent falls, foot deformities, scoliosis, distal muscular atrophy and prominent sensory ataxia. All patients and relatives were aware of the investigate nature of the studies and gave their consent.

HMSN-Lom mutation analysis

Screening for the p.R148X mutation was carried out in at least one patient of each family by amplification of the coding and flanking regions of exon 7 of the *NDRG1* gene and restriction analysis with *Taq*I as previously described [9].

Linkage and haplotype analyses

Linkage analysis was performed under the assumption of autosomal recessive inheritance, full penetrance, and equal frequencies of marker alleles. Pairwise LOD scores were calculated using the MLINK program version 5.1 of the FASTLINK package 2.1 [16]. Linkage analyses were carried out in those families that accomplished at least one of the two requirements: multiple affected children or parental consanguinity, having healthy parents. In any case, we also generated haplotypes for every proband and family.

To study HMSN-Russe locus on chromosome 10q23, five STR markers were selected, cen_*D10S1646-D10S210-D10S1647-D10S1672-D10S560*_tel, spanning a 2.9 Mb genomic region. To refine the critical interval shared, two

additional STR markers, *D10S2480* and *D10S1678*, and seven SNPs, rs874556, rs5030948, rs953724, rs906219, rs5030972, rs4746828, rs2394529, located within this 2.9 Mb region were analysed. STR markers were selected from previous reports [10, 11] and SNPs were ascertained from public databases (UCSC Genome Bioinformatics, http://genome.cse.ucsc.edu).

To study CMT4C locus on chromosome 5q23-q33 six microsatellite markers and one intragenic SNP of *SH3TC2* gene were analysed: cen_*D5S658-D5S1480-D5S413*-IVS14+69C/T-*D5S2015-D5S636-D5S820*_tel spanning a region of 17.6 Mb. Polymorphic microsatellites were selected from public databases (UCSC Genome Bioinformatics, http://genome.cse.ucsc.edu) and according to previous studies [17]. The SNP IVS14+69C/T is an intragenic polymorphism located in intron 14 on the *SH3TC2* gene.

Mutation analysis of the *SH3TC2* gene

Each coding exon and flanking intronic sequences of the *SH3TC2* gene were amplified from genomic DNA, with the use of overlapping primers as described elsewhere [17]. Mutation screening was performed by direct sequencing of purified PCR products (Quiagen, Hilden, Germany) in an ABI Prism 3100 autoanalyser (Applied Biosystems, Foster City, CA) by using fluorescent dideoxynucleotides and one of the PCR primers. All sequences on both strands were determined.

Dating the mutation

To estimate the original date of the mutation p.R1109X two mathematical approaches were applied: a Monte Carlo likelihood method implemented in the

program BDMC21 v2.1 [18; http://www.rannala.org/labpages/software.html] and a Markov chain method by means of the DMLE+ v2.2 software [19-21; http://www.dmle.org]. For both approaches, we used a generation time of 25 years and a proportion of population sampled of 0.082 (assuming a carrier frequency of CMT4C in Gypsy population= 2/100, which is a roughly estimation). The program BDMC21 v2.1 relies on the assumption that genetic variation among a group of highly linked polymorphic markers, defining a haplotype on which a novel non-recurrent mutation arose, is a function of the mutation frequencies of those linked markers and the time since the first occurrence of this unique mutation. To achieve this approach, we considered information from multiallelic STRs *D5S1480* and *D5S2015* that are the closest markers analysed to the small invariable core 3-C. Confidence interval was estimated following the standard theory of maximum likelihood estimation [22]. The second analysis performed was using the DMLE+ program version 2.2, which takes into account the marker information from the entire haplotype on the basis of cen_*D5S1480*-*D5S413*-IVS14+69C/T-*D5S2015*-*D5S636*_tel. This program allows Bayesian inference of the mutation age based on the observed linkage disequilibrium at multiple genetic markers.

Results

Up to date the p.R148X mutation in the *NDRG1* gene is the unique mutation associated with HMSN-Lom patients. Thus, as a first step, we searched for this mutation in every proband from the 20 Gypsy families included in the genetic

study. We detected the mutation in 4 out of 20 probands. When the p.R148X mutation was characterised in the proband, we confirmed that the mutation segregated in agreement with the disease status in each family, occurring in homozygous state in the affected patients while the parents were heterozygous, and unaffected sibs were either heterozygous or homozygous for the wild-type allele.

Once the HMSN-Lom founder mutation was excluded in 16 out of 20 families, we performed linkage and haplotype analyses with associated markers on the HMSN-Russe locus on chromosome 10q23. We excluded the HMSN-Russe locus in 12 families because either the lod score was lower than -2 and/or the disease did not segregate properly (data not shown). In order to obtain further information from extended haplotypes we typed four non-excluded families for two additional STR markers, *D10S2480* and *D10S1678*, and seven SNPs located within the 2.9 Mb region. We investigated allelic heterogeneity of these SNPs in a sample from the general Spanish population and we found that only SNP rs874556, mapped between STRs *D10S1647* and *D10S1672* was informative (data not shown). The four family probands maintained homozygosity for the entire haplotype suggesting possible linkage to the HMSN-Russe locus. In three families, CMT-444, CMT-42 and CMT-502, the disease segregated with the same haplotype while the patient of the family CMT-409 showed a different completely haplotype (Fig. 1). Thus, if we accept the hypothesis that HMSN-Russe gene is the causing gene of CMT in these families, present findings suggest that more than one mutation segregate in the families. Taking as a whole we demonstrated that 4 out of 20 families were HMSN-Lom and other 4 families could be associated with HMSN-Russe locus.

Twelve families associated with neither HMSN-Lom nor HMSN-Russe loci were available for further genetic studies.

The thirteen families supervised at the Department of Neurology of the La Fe Hospital in Valencia showed homogeneous clinical picture. The phenotype of most of patients from these families was similar to that of reported for CMT4C [8], caused by mutations in the *SH3TC2* gene located on chromosome 5q [17]. Thus, we decided to carry out linkage and haplotype analyses on CMT4C locus in 9 out of these 13 families. Four families were discarded because we previously demonstrated the HSMN-Lom mutation or linkage to the HSMN-Russe locus (Fig. 1). Then, we performed linkage and haplotype analyses of CMT4C locus in the 9 selected families. Pairwise analysis indicated linkage to CMT4C (Table 1). Patients from every family but one (CMT-421) carried a common core haplotype 3-C for markers *D5S413*-IVS14+69C/T. In seven families patients were homozygous for this haplotype (chromosomes D to Q; Fig. 2) whereas in one family we observed segregation of two different haplotypes with the disease (chromosomes A to C; Fig. 2). This finding suggested that two mutations in the *SH3TC2* gene were causing the disease, one of them being associated with the common haplotype that segregated in the families. Mutation analysis confirmed that all homozygous patients for the core haplotype were also homozygous for the p.R1109X (c.3325C>T) change. By contrast, patients from family CMT-235, for which we detected another haplotype segregating with the disease besides of the common haplotype (Fig. 3), were heterozygous for the p.R1109X mutation at exon 14; thus, we sequenced the remaining exons of the *SH3TC2* gene. We found a deletion of three cytosines in exon 11 that predicted a stop codon and a

truncated protein, p.C737_P738delinsX (c.2211-2213delCCC). So that, each mutation segregated with a different haplotype in family CMT-235 what would indicate that the disease shows allelic heterogeneity (Fig. 2; Fig. 3).

According to our results, the *SH3TC2* gene is causing the disease in 8 out of 9 families included in the CMT4C study. In family CMT-421, both loci HSMN-Lom and HSMN-Russe were discarded and although presented slightly linkage to the CMT4C locus, did not show the common haplotype and mutation analysis of exons 10, 11, 12 and 14 of the *SH3TC2* gene was negative (data not shown). So disease could be caused by a different mutation in the *SH3TC2* gene or by an unknown gene.

Once we found that CMT4C was the genetic diagnosis of 8 families of the clinical series from Valencia, we decided to perform genetic screening of CMT4C locus to the four families with possible linkage to HSMN-Russe locus (Fig. 1) and three families recruited from other Spanish regions (families VAL-9, VAL-15 and VAL-21) for which HSMN-Lom had been discarded and no linkage to HSMN-Russe locus could be demonstrated. Linkage, haplotype and mutation (exons 10, 11, 12 and 14 of *SH3TC2* gene) analyses suggested that CMT4C locus was not responsible for the disease in 5 out of 7 families. CMT-409 family, prior assigned to the HMSN-Russe locus, and VAL-21 family, were homozygous for the p.R1109X mutation (Fig. 2). As a whole the p.R1109X mutation was identified in 20 chromosomes (chromosomes B to U; Fig. 2) associated with the CMT4C disease in 10 out of 20 families.

Further we proceeded to estimate the original date of p.R1109X mutation in this Roma people. For that, we performed two mathematical approaches, the BDMC21 v2.1 program and the DMLE+ v2.2 software, and these analyses

showed us that the p.R1109X mutation is approximately 8.6 generations (95% CI 7.76-9.64 generations) and 8.7 generations (95% CI 7.3-11.3 generations), respectively. Both methods revealed similar estimations what would indicate, assuming a generation time of 25 years that the p.R1109X mutation probably arose about 200 years ago, by the end of the XVIIIth century and the beginning of the XIXth century according to the population data included in this study.

Discussion

Demyelinating or axonal autosomal recessive CMT, designated as CMT4, is genetically heterogeneous with twelve loci and ten genes identified so far [23]. Two of the CMT4 forms, HMSN-Lom [6, 7] and HMSN-Russe [10, 11] have been exclusively characterised in the European Gypsy population. We address the genetics of CMT in a series of 20 Gypsy unrelated families from Spain and we found ten families where disease is caused by mutations in *SH3TC2* gene at CMT4C locus; moreover, we found four HMSN-Lom families and three families with possible linkage to HMSN-Russe. In three families we could not establish any genetic association. All these data confirmed the high genetic heterogeneity of CMT neuropathy in Roma people from Spain.

Finding Spanish families that show the mutation p.R148X as responsible for HMSN-Lom is not striking since at least one HMSN-Lom Spanish family has been reported before [24, 25], and like in other European countries, HMSN-Lom is caused by the same mutation in our Gypsy population. Our data confirm those previously reported that pointed that the mutation p.R148X must be an

ancient single mutation whose origin predates the divergence of the first Gypsy communities because of its wide distribution across Europe [6, 9, 25].

We have also observed three families with possible linkage to HMNS-Russe in which the disease segregated with the same haplotype (Fig. 1). This haplotype presents a large similarity with those previously reported in HMSN-Russe families from Romania, France and Spain [10-12] what unambiguously points to a common origin and founder effect, similarly to that of HMSN-Lom. The conserved HMSN-Russe haplotype shares some aspects with HMSN-Lom and other Mendelian disorders associated with the Gypsy population [5]: diversity of haplotypes generated by numerous historical recombinations, with a small conserved region what highlights the old age of the mutation.

We have also demonstrated that the CMT is mainly caused by mutations located on the *SH3TC2* gene responsible for CMT4C [17] in 10 out of 20 cases. Patients from 9 out of 10 families carried the p.R1109X mutation in a homozygous state, while patients from one family were compound heterozygous, p.R1109X/p.C737_P738delinsX. We have also observed that p.R1109X is associated with a conserved haplotype what led us to think that these mutant chromosomes may have a common origin. This possible founder effect caused by the p.R1109X mutation has also been recently observed by Gooding et al. [26] on the basis that they have found the same mutation in two families (one from Spain and another from Turkey), besides of two additional unrelated Spanish affected subjects. This event would represent the fourth founder effect associated with demyelinating peripheral neuropathy in the European Gypsy population.

Many of Mendelian disorders, including this new described founder event [26], caused by private Gypsy mutations have been described as ancient mutations present in the Gypsy population prior to its diaspora in Europe and therefore shared by affected individuals throughout Europe [5]. This has been supported by the diversity of haplotypes around a small conserved core and its wide geographical distribution [5, 27]. Our findings have shown that 20 out 21 chromosomes associated with the disease carry the ancestral core haplotype 3-C that spans 113.6 Kb. Considering the interval comprised between the markers *D5S1480* and *D5S636* (approximately 5.5 Mb), haplotypes show slightly differences (Fig. 3) that could be easily explained by recombinations and instability of microsatellite alleles. On the other hand, nine of the CMT4C families reside in Valencia and 1 in Andalusia (Eastern and Southern Spain, respectively). Thus, on the basis of the haplotype size and their limited current geographical distribution, p.R1109X mutation may be generated by a relatively recent local founder mutation in our Gypsy population. In fact, some previous reports have described other founder effects specific for Gypsy population from Spain [28, 29].

To further investigate the genetic history of the p.R1109X mutation in our Gypsy population, we estimated the age of the mutation by two different mathematical approaches that revealed a similar number of generations: 8.6 (95% CI 7.76-9.64) and 8.7 (95% CI 7.3-11.3). Assuming a generation time of 25 years, the p.R1109X mutation probably arose by the end of the XVIIIth century and the beginning of the XIXth century (200 years ago). Morar et al [27] using disease haplotype coalescence times at different loci estimated that the Gypsy population was founded approximately 32-40 generations ago with

secondary and tertiary more recent founder events (approximately 16-25 generations ago). Our data suggest that the p.R1109X mutation would fall into the most recent group and it may be the consequence of a recent founder effect. By contrast, Gooding et al. [26] found the mutation in Spanish and Turkish families and they interpreted that the mutation was extremely ancestral, predating the commonly accepted date of migration of Gypsies out of Northern India. This idea is supported not just because p.R1109X mutation has been characterised in Spanish and Turkish individuals but also because of the small size of the conserved haplotype. Two plausible explanations could be glimpsed: a recurrent mutation in a CpG dinucleotide or a recent bottleneck in the Gypsy population in Spain. The first option is supported because the nucleotide change c.3325C>T that leads to the p.R1109X mutation is placed on a CpG dinucleotide and this is a hotspot for mutation in vertebrate genomes being CpG>TpG transitions the most common type of pathogenic mutations [30]. In such a case, the p.R1109X mutation might be detected in both Gypsy subjects and other CMT patients with a wide geographical distribution. According to our data a second alternative is more plausible: it is possible that a small number of related migrants split from a caste or tribal group carrying the p.R1109X mutation are the origin of the current distribution in Spain. They could have played the role of a bottleneck in our Gypsy population [27, 31]. Thus, when the origin of the p.R1109X mutation is studied in our population, we would only considerer data since this bottleneck happened. Moreover, maybe both ideas are right in certain degree and in the current days results described in Gypsy population are consequences in large or small extent of both events as previously reported for other cases [32].

With the exception of phenylketonuria, in the Gypsy population Mendelian disorders have been described as genetically homogenous with a private founder mutation causing disease in all patients [5]. We have also characterised another mutation in a heterozygous state causing CMT4C in a Gypsy family, p.C737_P738delinsX. In the same sense, none of the two mutations characterised in Gypsy patients have been also detected in Caucasian population. In this last population several mutations have been described on the *SH3TC2* gene showing quite mutational heterogeneity [17]. Further studies with a wider sampling are necessary to determine if both populations share some mutation likely due to admixture like happens with cystic fibrosis and ΔF508 [33] or phenylketonuria and the p.R252W and IVS10nt546 mutations [34, 35]. It is tempting to speculate that the p.R1109X mutation is a private founder mutation exclusive for Gypsy population and the p.C737_P738delinsX mutation has another different origin maybe introduced by admixture.

In summary, we have demonstrated that besides of the HMSN-Lom and HMSN-Russe loci, CMT4C locus seems to be responsible of most of Gypsy cases with a diagnosis of a demyelinating CMT4. To date, the p.R1109X mutation in the *SH3TC2* gene is implicated in the developing of the disease in a homozygous or a compound heterozygous state and likely as a rare disease, originated once in the human history. Other mutations in the *SH3TC2* gene are also described in other populations what emphasises the complexity associated with the genetics of CMT. Moreover, the known peripheral neuropathies associated with Gypsies do not explain all cases of CMT4 in this population and therefore, additional forms remain to be characterised.

Table 1 Combined two-point lod scores for analysed markers at CMT4C locus

MARKERS	RECOMBINATION (θ)						
	0.0	0.01	0.05	0.1	0.2	0.3	0.4
D5S658	-∞	1.11	2.19	2.61	1.96	1.10	0.39
D5S1480	3.26	3.86	3.93	3.51	2.42	1.32	0.53
D5S413	8.01	7.81	7.00	5.98	3.98	2.18	0.78
D5S2015	4.36	4.91	4.84	4.27	2.85	1.53	0.54
D5S636	4.71	5.26	5.19	4.61	3.17	1.75	0.65
D5S820	-1.55	0.27	1.60	1.82	1.44	0.76	0.19

LEGENDS

Fig. 1 Families with possible linkage to the HMSN-Russe locus. Every affected individual is homozygous for the entire haplotype. In families CMT-444, CMT-42 and CMT-502 patients share the same haplotype for all the markers except for *D10S1647* and rs874556. In family CMT-409 the patient shows a rather different haplotype. All these families belong to the clinical series supervised at La Fe Hospital in Valencia except CMT-42 that comes from Madrid. (SYMBOLS: Haplotypes associated with the disease are indicated with black bars in all the cases, although they show some allele differences. Haplotypes non-associated with the disease are shown with lined barcodes.)

Fig. 2 Haplotypes constructed for markers at CMT4C locus. Mutations in the *SH3TC2* gene borne by every chromosome are also shown in the last column. All these families belong to the clinical series supervised at La Fe Hospital in Valencia except VAL-21 from Andalusia. Family CMT-409 was thought to be a HMNS-Russe case at first. The shaded area designates the assumed conserved haplotype where it is noticeable that the small core haplotype 3-C is always present when the p.R1109X mutation is present.

Fig. 3 Family CMT-235 linked to CMT4C locus. Two different haplotypes were observed segregating with the disease. Haplotype in black bars is the common one seen in the other families linked to the CMT4C locus and is associated with the founder mutation p.R1109X (c.3325C>T). Haplotype with grey bars was only found in this family and is associated with the mutation p.C737_P738delinsX (c.2211-2213delCCC). (SYMBOLS: Lined bars represent chromosomes non-associated with the disease. Blackened and greyish bars show chromosomes associated with the disease and therefore carrying a mutation.)

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