Short Report

Genetics of the Charcot-Marie-Tooth disease in the Spanish Gypsy population: the Hereditary Motor and Sensory Neuropathy-Russe in depth

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A**bstract**

Four private mutations responsible for three demyelinating forms of Charcot-Marie-Tooth (CMT) or hereditary motor and sensory neuropathy (HMSN) have been associated with the Gypsy population: the *NDRG1* p.R148X involved in CMT type 4D (CMT4D/HMSN-Lom); p.C737_P738delinsX and p.R1109X mutations in the *SH3TC2* gene responsible for CMT type 4C (CMT4C); and a G>C change in a novel alternative untranslated exon in the *HK1* gene causative of CMT type 4G (CMT4G/HMSN-Russe). Here we address the findings of a genetic study of 29 Gypsy families with autosomal recessive demyelinating CMT from Spain. The most frequent form is CMT4C (57.14%), followed by HMSN-Russe (25%) and HMSN-Lom (17.86%). The relevant frequency of HMSN-Russe has allowed us to investigate in depth the genetics and the associated clinical symptoms of this CMT form. HMSN-Russe probands share the same haplotype confirming that the *HK1* g.9712G>C is a founder mutation, which would arrive in Spain around the end of the 18th century. From the phenotype and course of disease we can conclude that HMSN-Russe is characterized by a marked distal atrophy and preservation of motor nerve conduction velocities in presumably unaffected nerves which suggest that primary axonal disorder probably plays an important role in pathogenesis.

Key words: Charcot-Marie-Tooth disease; Gypsy population; Hereditary Motor and Sensory Neuropathy type Russe; founder mutation.

Introduction

The Spanish Gypsy (Roma) population represents one of the largest communities in Europe (1) and make up around 1.5% of the total Spanish population (500,000 – 600,000 individuals) (http://www.unionromani.org). Due to the Gypsy population is a closed community a number of private disease-causing mutations are inherited as an autosomal recessive trait and usually with evidence of a founder event. To date three recessively inherited demyelinating Charcot-Marie-Tooth (CMT) or hereditary motor and sensory neuropathy (HMSN) forms have been associated with Gypsies: (i) CMT type 4D (CMT4D)/HMSN-Lom (MIM 601455) due to the p.R148X mutation in the *NDRG1* gene (2); (ii) CMT type 4C (CMT4C; MIM 601596), which could be caused by the p.C737_P738delinsX and/or p.R1109X mutations in the *SH3TC2* gene (3); (iii) and CMT type 4G (CMT4G)/HMSN-Russe (MIM 605285), which is associated with a G>C change in a novel alternative untranslated exon (AltT2) in the *HK1* gene (4). HMSN-Lom and CMT4C are forms widely documented in populations from different origins (2, 5-8), whereas HMSN-Russe associated phenotype has only been reported in detail by Thomas et al. (9).

Here we report the genetic findings of a clinical series of 29 Gypsy families from Spain which present with an autosomal recessive demyelinating CMT. After CMT4C, the second cause of CMT in Spanish Gypsies is HMSN-Russe. This relevant prevalence has allowed us to investigate the clinical features of this severe CMT form and to follow-up 11 affected individuals from 7 families with the aim to define better the progression of HMSN-Russe.

Materials and Methods

Patients

Our clinical series comprises 29 unrelated Spanish Gypsy probands diagnosed with demyelinating CMT neuropathy. Some genetic data belonging to 17 of these families were previously reported (3). Families are from several Spanish regions: 18 families live in Valencia, 6 in Andalusia, 3 in Catalonia, 1 in Extremadura and 1 in Murcia. All protocols performed in this study complied with the ethics guidelines of the institutions involved. All patients and relatives were aware of the investigative nature of the studies and gave their consent.

Clinical, electrophysiological and histological assessments

Eleven patients affected by HMSN-Russe belonging to 7 unrelated families were examined. The clinical assessment included strength, muscle atrophy, sensory loss, reflexes, foot deformities as well as a general and neurologic examination. Muscle strength was graded using the standard Medical Research Council (MRC) scale. CMT neuropathy score (CMTNS) was applied to determine neurological impairment: mild (CMTNS ≤10 points), moderate (CMTNS 11–20), and severe (CMTNS 21–36) (10). The functional disability scale (FDS) was used to measure the disability (11). Electrophysiological studies were performed in 11 of the patients following the same protocol that was previously described (12). Sural nerve biopsy was performed in the patient CMT502-524 when he was 15 years old as previously described (12, 13).

Genetic studies

With the aim to investigate if our patients were carriers of more of one mutation, since the Gypsy population has a high rate of endogamy, we have analyzed the four mutations

exclusively associated with Gypsies involved in CMT in the 29 probands. First, we performed a screening of the *NDRG1* p.R148X mutation, and of the p.C737_P738delinsX and p.R1109X mutations in the *SH3TC2* gene reported elsewhere (2, 3, 14, 15). Next, we investigated the HMSN-Russe locus by haplotype analyses with four STR (Single Tandem Repeat) markers, cen_*D10S1646-D10S210-D10S2480-D10S1678*_tel, and by analyzing the putative mutation reported in the *HK* gene (4) which we have named as *HK1* g.9712G>C according to the reference sequence NM_033498 following the recommendations of the Human Genome Variation Society (16). Finally, we estimated the allelic age of the *HK1* g.9712G>C mutation using the program BDMC21 v2.1 (http://www.rannala.org/docs/bdmcdoc.html) as previously described (17).

Results

Mutational screening, haplotype analysis and founder effect

In the whole series reported here, we have found that 5 (17.24%) index cases were HMSN-Lom (homozygous for the *NDRG1* p.R148X mutation), 7 (24.14%) probands were HMSN-Russe (homozygous for the *HK1* g.9712C mutation), and 17 (58.62%) were CMT4C (13 probands homozygous for *SH3TC2* p.R1109X, 2 compound heterozygous for *SH3TC2* p.C737_P738delinsX/p.R1109X, 1 compound heterozygous for *SH3TC2* p.R1109X/p.R954X, and 1 homozygous for *SH3TC2* p.C737_P738delinsX). Consequently the disease-causing mutation has been identified in all our patients and all of them harbored mutations in only one of the investigated genes, except proband CMT148-485 a female patient homozygous for the *SH3TC2* p.R1109X and heterozygous for the *HK1* g.9712G>C mutation.

The HMSN-Russe is the second cause of CMT in our Spanish Gypsy series, which is due to a founder mutation since our 7 HMSN-Russe probands and other previously reported patients share the same haplotype (Table 1) (9, 17, 18). We estimated that the *HK1* g.9712G>C dates back approximately 10.0 generations (95% CI: 8.34-11.66 generations) (Figure 1). Assuming a 25-year generation span, the *HK1* g.9712G>C mutation probably already was in Spain 250 years ago, around the end of the 18th century, according to the population data included in this study.

Clinical picture

Clinical features of 11 affected individuals from 7 families suffering from HMSN-Russe are compiled in Table 2. Distal lower and upper limb weakness was present in all patients. Over time, the weakness extended to proximal lower limb muscles in half of cases. The onset was in childhood in most cases, being two of them around adolescence. Distal sensory loss and areflexia were present in every patient. Foot deformity mainly pes cavus was present in all the cases and scoliosis was found in 5/11 cases. The majority of patients walk with difficulties, 4 of them need ankle foot orthosis and the older one was wheelchair dependent. No values were obtained for motor nerve conduction velocity (MNCV) in the median nerve in two patients recording from small hand muscles. MNCV values were in the demyelinating range, between 20 and 32 m/s; in the oldest two patients (CMT131-409 and CMT155-549) distal motor nerves were unexcitable. Nerve conduction in a presumably unaffected nerve (axillary) was performed on the patient CMT502-971, resulting distal latency in the normal range (Table 2). Sensory nerve action potentials were not detectable in 5 patients. Sural nerve biopsy performed in the patient CMT502-524 showed the absence of hyperthrophic

changes, and the profuse presence of small-caliber fibers and clusters of regenerative fibers (Figure S1).

It should be noted that the patient CMT131-411 with the earliest onset also manifests additional signs (severe mental retardation with severe language impairment). CMT131-411 is born to a double consanguineous parents and his mother (CMT131-409) is also affected by HMSN-Russe. He has a brother who is a heterozygous carrier of the *HK1* g.9712G>C and shows normal MNCV although he also suffers similar additional manifestations. In the aggregate, these manifestations suggest that CMT131-411 can suffer from other condition with genetic bases not yet diagnosed besides of the HMSN-Russe. These unknown genetic bases would explain the complex phenotype observed in these patients.

Discussion

The analysis of the four CMT mutations (*NDRG1* p.R148X, *SH3TC2* p.C737_P738delinsX and p.R1109X, and *HK1* g.9712G>C) exclusively associated with Gypsies has allowed us to identify the disease-causing mutation in all the 29 Spanish families. The most frequent CMT form in Spanish Gypsies is CMT4C, being the p.R1109X mutation the most predominant one (3). The *SH3TC2* p.C737_P738delinsX has only been identified in Gypsy patients from Spain, and together with the *FANCA* p.Q99X involved in Fanconi anemia (MIM 227650) (19) are the only founder mutations specifically identified in Spanish Gypsies. The *SH3TC2* p.C737_P738delinsX is probably a private mutation of recent origin not representative of the overall molecular pattern of CMT in the Gypsy population. Moreover, one proband harbored in heterozygous the *SH3TC2* p.R954X mutation, which is a widely reported change in

several non-Gypsy populations (6-8) and even, is considered a founder effect in the French-Canadian population (20). This event is probably the consequence of admixture with neighboring populations, since his grandmother could have been a non-Gypsy Caucasian woman.

The second cause of demyelinating CMT in Spanish Gypsy population is the HMSN-Russe (24.14%) followed by HMSN-Lom (17.24%). To further investigate the genetic history of the founder mutation *HK1* g.11027G>A associated with the HMSN-Russe form, we estimated that the common ancestor carrier of this mutation lived at the end of the 18th century (~10 generations ago). We obtained similar results for the *SH3TC2* p.R1109X mutation, which was dated at ~9.1 generations before the present (3). We postulate that due to their wide distribution in Europe (1, 3, 14), both mutations (*HK1* g.9712G>C and *SH3TC2* p.R1109X) may be ancient founder events previous to the Gypsy diaspora from India. These mutations could have arrived in Spain around the end of the 18th century as a consequence of a population split from a tribal group that can be regarded as a secondary bottleneck. In 1783, Charles III of Spain broke with repressive legislation against Gypsies carried out by Spanish Monarchy and gradually, favored an increasing of the settlements of Gypsy population. Thus, one of our probands, CMT148-485, is homozygous for the *SH3TC2* p.R1109X mutation and heterozygous for the *HK1* g.9712G>C which supports the hypothesis that both mutations arrived in Spain from the same Gypsy group. The present study demonstrates the potential of dating founder mutations as a way to gain insight into the genetic history of disease and help us to understand the genetic diversity of populations.

The complete description of the HMSN-Russe phenotype made by Thomas et al.(9) showed the main clinical manifestations of this neuropathy that we have

observed in our patients as well. From the phenotype and course of disease in patients in our series, we can draw that HMSN-Russe is a severe neuropathy, motor and sensory with prominent distal muscle atrophy that spread to proximal lower limb after adolescence; adult patients show severe disability assessed according to CMT FDS. So marked distal atrophy and preservation of MNCV in presumably unaffected nerves suggest that primary axonal disorder probably plays an important role in pathogenesis. The possibility that axons, rather than Schwann cells (SCs) may be the primary target of the disease process is open. HK1 activity is not altered in cultured HMSN-Russe SCs and nerve biopsy immunohistochemistry shows no differences between HMSN-Russe and healthy individuals (4). Further studies are necessary to provide a straightforward indication of the origin of the primary defect.

In conclusion, we have reported here a complete genetic history of the three CMT forms associated with Gypsies in the Spanish population. The HMSN-Russe, the second more frequent CMT in our Gypsy population, is a severe neuropathy which progressively leads to a notable disability characterized by a marked distal atrophy and preservation of motor nerve conduction velocities in presumably unaffected nerves. HMSN-Russe would be due to an ancestral founder event present at the time of the founding Gypsy population and would arrive in Spain around the end of the 18th century together with the disease-causing mutation involved in CMT4C.

References

1. Kalaydjieva L, Gresham D, Calafell F. Genetic studies of the Roma (Gypsies): a review. BMC Med Genet 2001: 2: 5.

2. Kalaydjieva L, Gresham D, Gooding R et al. N-myc downstream-regulated gene 1 is mutated in hereditary motor and sensory neuropathy-Lom. Am J Hum Genet 2000: 67: 47-58.

3. Claramunt R, Sevilla T, Lupo V et al. The p.R1109X mutation in SH3TC2 gene is predominant in Spanish Gypsies with Charcot-Marie-Tooth disease type 4. Clinical Genetics 2007: 71: 343-349.

4. Hantke J, Chandler D, King R et al. A mutation in an alternative untranslated exon of hexokinase 1 associated with hereditary motor and sensory neuropathy -- Russe (HMSNR). Eur J Hum Genet 2009: 17: 1606-1614.

5. Kalaydjieva L, Nikolova A, Turnev I et al. Hereditary motor and sensory neuropathy--Lom, a novel demyelinating neuropathy associated with deafness in gypsies. Clinical, electrophysiological and nerve biopsy findings. Brain 1998: 121 (Pt 3): 399-408.

6. Senderek J, Bergmann C, Stendel C et al. Mutations in a gene encoding a novel SH3/TPR domain protein cause autosomal recessive Charcot-Marie-Tooth type 4C neuropathy. Am J Hum Genet 2003: 73: 1106-1119.

7. Azzedine H, Ravise N, Verny C et al. Spine deformities in Charcot-Marie-Tooth 4C caused by SH3TC2 gene mutations. Neurology 2006: 67: 602-606.

8. Houlden H, Laura M, Ginsberg L et al. The phenotype of Charcot-Marie-Tooth disease type 4C due to SH3TC2 mutations and possible predisposition to an inflammatory neuropathy. Neuromuscul Disord 2009: 19: 264-269.

9. Thomas PK, Kalaydjieva L, Youl B et al. Hereditary motor and sensory neuropathy-russe: new autosomal recessive neuropathy in Balkan Gypsies. Ann Neurol 2001: 50: 452-457.

10. Shy ME, Blake J, Krajewski K et al. Reliability and validity of the CMT neuropathy score as a measure of disability. Neurology 2005: 64: 1209-1214.

11. Birouk N, Gouider R, Le Guern E et al. Charcot-Marie-Tooth disease type 1A with 17p11.2 duplication. Clinical and electrophysiological phenotype study and factors influencing disease severity in 119 cases. Brain 1997: 120 (Pt 5): 813-823.

12. Sevilla T, Cuesta A, Chumillas MJ et al. Clinical, electrophysiological and morphological findings of Charcot-Marie-Tooth neuropathy with vocal cord palsy and mutations in the GDAP1 gene. Brain 2003: 126: 2023-2033.

13. Sivera R, Espinos C, Vilchez JJ et al. Phenotypical features of the p.R120W mutation in the GDAP1 gene causing autosomal dominant Charcot-Marie-Tooth disease. J Peripher Nerv Syst 2010: 15: 334-344.

14. Gooding R, Colomer J, King R et al. A novel Gypsy founder mutation, p.Arg1109X in the CMT4C gene, causes variable peripheral neuropathy phenotypes. J Med Genet 2005: 42: e69.

15. Hunter M, Bernard R, Freitas E et al. Mutation screening of the N-myc downstream-regulated gene 1 (NDRG1) in patients with Charcot-Marie-Tooth Disease. Hum Mutat 2003: 22: 129-135.

16. den Dunnen JT, Antonarakis SE. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Hum Mutat 2000: 15: 7-12.

17. Claramunt R, Pedrola L, Sevilla T et al. Genetics of Charcot-Marie-Tooth disease type 4A: mutations, inheritance, phenotypic variability, and founder effect. J Med Genet 2005: 42: 358-365.

18. Rogers T, Chandler D, Angelicheva D et al. A novel locus for autosomal recessive peripheral neuropathy in the EGR2 region on 10q23. Am J Hum Genet 2000: 67: 664-671.

19. Callen E, Casado JA, Tischkowitz MD et al. A common founder mutation in FANCA underlies the world's highest prevalence of Fanconi anemia in Gypsy families from Spain. Blood 2005: 105: 1946-1949.

20. Gosselin I, Thiffault I, Tetreault M et al. Founder SH3TC2 mutations are responsible for a CMT4C French-Canadians cluster. Neuromuscul Disord 2008: 18: 483-492.

Figure 1 Maximum likelihood curves obtained by means of BDMC21 v2.1. Graphic shows the log-likelihood values and the corresponding number of generation estimates regarding the time the *HK1* g. 9712G>C mutation arrived in Spain.

Figure S1 Semithin transverse section through sural nerve showing reduced large fibers and regenerative clusters. Magnification x 40.

Table 1 Haplotypes constructed for markers on the HMSN-Russe locus

ID no. Family	ID no. Proband	Origin	Case type	Haplotype based on cen_D10S1646-D10S210-D10S2480-D10S1678_tel			
CMT502[a]	524	Valencia	Familial	2	5	1	2
CMT444[a]	514	Valencia	Sporadic	2	5	1	2
CMT260	704	Catalonia	Sporadic	2	5	1	2
CMT198	630	Valencia	Sporadic	2 4	5	1	2
CMT288	804	Catalonia	Sporadic	3	5	1	2
CMT155	549	Andalusia	Familial	3 4	5	1	2
CMT131	410	Andalusia	Sporadic	2 3	5	1	2
CMT-148[b]	485	Valencia	Familial	3 5	4 5	2 1	2

[a] Families classified as probable linkage to HMSN-Russe locus in Claramunt et al (3).
[b] Proband CMT148-485 harbors the *HK1* g.9712G>C mutation in heterozygosis.
The shaded area designates the assumed conserved haplotype.

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Table 2 Clinical summary of HMSN-Russe patients

ID no. Family	ID no. Patient	Age (y)		Weakness			Sensory loss	Pes cavus	Scoliosis	NDS	FDS	MNCV median (m/s) [y]	CMAP median (mV)	SNAP
		at onset	at last exam	UL	LL	Prox								
CMT198	630	Infancy	35	++	+++	No	P/V/T	Yes	No	16	3	32 [24y]	7	NR
CMT444	514	<4y	17	+	+++	Yes	P/V/T	Yes	No	20	4	29 [10y]	5.8	3.4
CMT444	515	Infancy	16	?	++	?	P/V	Yes	Yes	22	4	22 [16y]	2	NR
CMT502	524	7	19	+	++	No	V	Yes	Yes	15	3	30 [14y]	7.7	1.5
CMT502	528	7	13	+	+++	Yes	V	Yes	Yes	15	4 AFO	ND	_	_
CMT502	971	7	22	++	+++	Yes	All	Yes	No	24	4 AFO	17.3[(a)][22y] [(b)]	0.1	1.6
CMT288	804	4	10	+	++	No	P/V	Yes	No	19	4 AFO	26	3.2	NR
CMT260	704	4	19	+	++	No	P/V	Yes	Yes	20	4 AFO	20.6	0.1	NR
CMT155	549	16	48	+++	+++	Yes	P/V/T	Yes	No	27	7	NR [46y]	NR	3.5
CMT131	410	1	13	+++	+++	Yes	P/V	Yes	No	22	4	27 [2y]	2.5	11
CMT131	409	10	37	+++	+++	No	All	Yes	No	22	4	NR [37]	NR	NR

NDS, neurological disability score; FDS, functional disability scale; MNCV, motor nerve conduction velocity; CMAP, compound motor action potential; SNAP, sensory nerve action potential; UL, upper limbs; LL, lower limbs; Prox, proximal; P, pinprick; V, vibratory; T, Touch; AFO, Ankle-Foot Orthesis; ND, not done; NR, not response.

[(a)]MNCV to proximal muscles: 33 m/s. [(b)]Axillary nerve latency 3.2 ms, # axillary nerve CMAP= 10.