Non-radioactive detection of 17p11.2 duplication in CMT1A: a study of 78 patients

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Abstract

Charcot-Marie-Tooth disease type 1 (CMT1) is a peripheral neuropathy characterised by progressive distal muscular atrophy and sensory loss with markedly decreased nerve conduction velocity, mostly inherited as an autosomal dominant trait. The most common form, type 1A, is associated with a 1.5Mb DNA duplication in region p11.2-p12 of chromosome 17 in many patients.

In this study a non-radioactive test for detection of the CMT1A duplication based on an RM11-GT microsatellite polymorphism is presented. Although different methods have been devised for this purpose, the present method has the advantage of being rapid, informative, economical, easily interpretable, and, therefore, it represents a very useful tool for diagnosis of CMT1A, especially before clear manifestation of clinical symptoms.

Seventy-eight patients diagnosed clinically as having CMT and evaluated by electrophysiological methods were tested with an RM11-GT microsatellite and with probe pVAW409R3. The CMT1A duplication was found in 76% of the 56 unrelated patients. RM11-GT was the most informative marker with a heterozygosity of 89%.

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Charcot-Marie-Tooth disease type 1 (CMT1) is the most common subtype of the hereditary motor and sensory neuropathies (HMSN).¹ It is characterised clinically by symmetrical distal muscle atrophy, slowly progressive weakness, and pes cavus deformity, electrophysiologically by reduced nerve conduction velocities (NCV) of both motor and sensory nerves, and histologically by segmental de- and remyelination patterns which can be observed on sural nerve biopsy.

CMT1 is a genetically heterogeneous disorder in which all forms of mendelian segregation have been reported.² The most common form, type 1A, is the result of a dominant allele mapping on chromosome 17³⁻⁶ associated with either a 1.5 Mb DNA duplication

in 17p11.2-p12⁷⁻⁹ including the peripheral myelin protein-22 (PMP-22) gene¹⁰⁻¹³ or of a point mutation of the gene itself.¹⁴⁻¹⁵ In a very small number of families (CMT1B) linkage to markers on chromosome 1q21-q23¹⁶⁻¹⁹ has been found. Two more families in which the disease segregates independently of the previous loci have been described, suggesting that a third locus may be involved.²⁰ Finally, an autosomal recessive form of CMT1 has been documented, although its location is still undefined.^{21,22}

The aim of this research was to set up a non-radioactive method for detection of the chromosome 17p11.2 duplication in CMT1A.

Materials and methods

PATIENTS

Seventy-eight CMT1 patients, living in different regions of Italy, were diagnosed at the Department of Neurology, University of Padova, at the Department of Neurology, University of Pisa, and at the Neurophysiology Department, Rizzoli Orthopaedic Institute, Bologna. The family pedigrees were reconstructed in order to establish the pattern of inheritance and to allow discrimination between familial and sporadic cases; whenever necessary biological paternity was tested by DNA markers.

In all subjects complete clinical and electrophysiological evaluation was carried out. The selection criteria, accepted by the European CMT Collaborative Research Group, for the affected subjects were the following: presence of slowly progressive symmetrical muscle wasting and weakness, predominantly of the distal lower limbs, severely decreased motor conduction velocity (median nerve conduction velocity (MCV) <30 m/s), absence or marked decrease of sensory nerve action potentials (SNAPs) in the lower limbs, or sensory nerve biopsy consistent with a diagnosis of demyelinating neuropathy.

DNA ANALYSIS

Two methods of genomic analysis were used: restriction fragment length polymorphism (RFLP) detection with DNA probes on Southern blot and microsatellite analysis by PCR. DNA extracted from venous blood samples

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(A) Silver stained electrophoretic separation of PCR products obtained with RM11-GT. 1-8 represent the RM11-GT alleles; the size range is 153-167bp. N=normal subject; B, D, E, G, I=patients with duplication visualised as different dosage of two alleles; A, C, H=patients with duplication visualised as three different alleles; F=patient without duplication. (B) Densitometric scanning of samples N (normal subject) and E (patient with duplication) from (A). The dotted line is the normal densitometer scanning; the dark line is the Gaussian interpolation (GS-370 Data System). * = Peak corresponding to the duplicated band.

was digested with *MspI*, separated by electrophoresis on 0.9% agarose gel, and transferred by Southern blotting to nylon membranes (Hybond N+, Amersham). A single copy fragment of the probe pVAW409R3 (D17S122) was labelled with ^{32}P dCTP by the random priming method. This probe detects three *MspI* alleles with sizes of 2.8, 2.7, and 1.9 kb; in affected subjects the duplication can be visualised as three different bands corresponding to three different *MspI* alleles or as two bands, one of them giving a stronger hybridisation signal for the presence of that *MspI* allele in two copies.^{23,24}

The RM11-GT genotype was determined by PCR under standard conditions using the GT strand (CAGAACCACAAAATGTCTTGC-ATTC) and the CA strand (GGCCAGACAGACCAGGCTCTGC) oligonucleotide primers flanking the (GT)_n repeat. Polymerase chain reaction was performed using 0.1 µg genomic DNA, 2.4 pmol/l of each primer, 160 mmol/l of each dNTP, 1 × reaction buffer

(67 mmol/l Tris-HCl, pH 8.8, 16.6 mmol/l (NH₄)₂SO₄, 1.5 mmol/l MgCl₂, 0.01% Tween-20), and 0.5 units of RTB polymerase (BIOLINE) per 25 µl reaction. After an initial denaturation for three minutes at 94°C, 24 cycles of amplification with denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55°C, and extension for 30 seconds at 72°C, followed by a 10 minute final elongation at 72°C, were performed in an MJResearch Programmable Thermal Controller. Amplification products were run on 8% acrylamide gels (1 mm thick, containing 10% glycerol). The gels were then silver stained according to Santos *et al.*,²⁵ with some modifications, and then dried by a BIORAD Gel Dryer.

The polymorphic (GT)_n marker, derived from pVAW409R1 and located at D17S122, shows eight different alleles in the 153-167 bp range.^{7,26,27} The duplication appears as either three different alleles or two different alleles one of which is present in two copies giving a stronger signal.

Dosage of alleles was determined by visual assessment and corroborated by transmission densitometry scanning. Densitometric data were processed according to Hu *et al.*²⁸; the area under each peak represents the intensity of each band. Each peak area was divided by the total peak area of the two bands in the individual sample lane to obtain the relative peak value, that is, the relative band intensity. Since bands with double intensity increase the total peak area and alter the relative peak value, a normalisation factor was applied. Bands showing single intensity in a normal subject and similar intensity in the suspected duplication case were selected. The ratio of the peak values of these bands for normal subject and for duplication case provides the appropriate normalisation factor. All the relative peak values from the duplication case were then multiplied by this factor.

Results

Seventy-eight CMT1 patients, belonging to 56 Italian families, were tested with an RM11-GT microsatellite and with probe pVAW409R3 (D17S122). A typical outcome of the silver stained electrophoretic separation is presented in the figure, along with the results of its densitometric scanning. The duplication could be visualised as three different alleles or different dosage of two alleles. This dosage was determined only in subjects who were heterozygous since the results were most reproducible and reliable in such cases.

Forty-four of the 56 index cases proved to be heterozygous both for the microsatellite RM11-GT and for probe pVAW409R3; six patients showed heterozygosity only for the RM11-GT microsatellite and one only for the pVAW409R3 (table). Heterozygosity was 89% for the (GT)_n marker and 80% for the pVAW409R3 probe. The presence of the CMT1A duplication was shown in the same 37 index cases by both markers. In six patients who were homozygous for the pVAW409R3 probe, information about the duplication was obtained by the RM11-

Comparison of results obtained with the RM11-GT microsatellite and with the pVAW409R3 probe in 56 unrelated CMT1A patients

Patients	Heterozygous subjects		Duplicated subjects		Presence of three alleles	
	RM11-GT	VAW409R3	RM11-GT	VAW409R3	RM11-GT	VAW409R3
1F	+	+	+	+		
2F	+	+	+	+	*	
3F	+	+	+	+		*
4F	+	+	+	+		
5F	+	+	+	+	*	
6F	+	+	+	+	*	
7F	+	+	+	+		*
8F	+	+	+	+	*	
9F	+	-	-	/		
10F	+	+	+	+	*	
11F	+	+	-	-		
12F	+	-	-	/		
13F	+	-	-	/		
14F	+	-	-	/		
15F	+	+	+	+		
16F	+	+	+	+		
17F	+	-	+	/		
18F	+	+	+	+	*	
19F	+	+	+	+		
20F	+	+	+	+		
21F	+	+	+	+		
22F	+	+	+	+	*	
23F	+	+	+	+		
24F	+	+	-	-		
25F	+	+	+	+		
26F	+	+	+	+		
27F	+	+	+	+		
28F	-	-	/	/		
29F	-	+	/	-		
30F	+	+	-	-		
31F	+	+	+	+	*	
32F	+	+	-	-		
33F	+	+	+	+	*	*
34F	+	+	-	-		
35F	+	+	+	+		
36F	+	+	+	+		
37F	+	+	+	+	*	
38F	+	+	+	+		
39F	+	+	+	+		
40F	+	+	+	+		
41F	+	+	+	+		
42F	-	-	/	/		
43F	+	+	+	+		
44F	+	+	+	+		
45F	+	+	+	+		
46S	+	+	+	+		
47S	+	+	+	+	*	*
48S	+	+	+	+		
49S	+	+	-	-		
50S	-	-	/	/		
51S	-	-	/	/		
52S	+	+	-	-		
53S	-	-	/	/		
54S	+	+	+	+		
55S	+	-	-	/		
56S	+	+	+	+		
Total	50	39	38	37	11	4

F = familial case. S = sporadic case. / = Samples in which the allele dosage test was not performed as they were not informative (homozygous) for the corresponding marker.

GT microsatellite; of these, five were found not to have the duplication while one had the duplication. However, one patient who was homozygous for the (GT)_n marker showed non-duplication when the pVAW409R3 probe was used.

The (GT)_n marker showed direct evidence of the duplication, indicating the presence of three alleles in 11 cases, while in only four CMT1A duplicated patients were all three alleles detected by the probe pVAW409R3 (table).

In total the duplication 17p11.2 was shown with pVAW409R3 or RM11-GT or both in 39 of the 51 heterozygous subjects giving a duplication frequency of 76%. In the other 22 CMT1A subjects, related to the 56 index cases, the two markers always showed the same genotype as the corresponding index case.

Neither any of the healthy subjects who have been tested as controls nor any of the parents of the sporadic cases showed evidence of duplication with both markers.

Discussion

Different methods are available for the detection of the CMT1A duplication, such as measurement of dosage differences between RFLP alleles by Southern analysis, detection of a junction fragment by pulsed field gel electrophoresis (PFGE), and analysis of metaphase chromosomes by in situ hybridisation.^{6,7,27,29,30} The microsatellite RM11-GT analysis by radioactive PCR is definitely the most rapid and informative.^{26,27} The non-radioactive analysis of the polymorphic (GT)_n marker presented in this study has the advantage of being not only rapid and informative but also economical and easily interpretable.

The microsatellite RM11-GT has a higher level of polymorphism than that of the pVAW409R3 probe and this makes it more likely to show three separate alleles. It allowed a direct and simple demonstration of the 17p11.2 duplication in 29% of the duplicated patients while the pVAW409R3 was informative in only 11% of the duplicated cases. Wise *et al.*²⁷ reported that RM11-GT can be considered informative for the presence of CMT1A duplication only when three alleles are detectable, because shadow bands make it difficult to assess dosage between two alleles. The detection of banding patterns by silver staining instead of radiolabelling methods allows this problem to be overcome.

In the present study RM11-GT showed 89% heterozygosity which is higher than that observed with pVAW409R3 (80%) and in general terms it is the highest level of heterozygosity so far detected in studies dealing with the detection of duplication in CMT1A.^{7,27}

To estimate the duplication frequency the number of affected subjects in which the duplication was shown with pVAW409R3 or RM11-GT or both was calculated on the total number of informative (heterozygous) patients. Therefore, the frequency of CMT1A duplication in this sample of unrelated affected subjects was 39/51, corresponding to 76% of the total. Wise *et al.*²⁷ found a 68% duplication frequency in a sample of comparable size. A previous study on a regional basis, performed by our group, estimated the duplication frequency to be 82%, with confidence limits from 73.8% to 94.8%.³¹

Given the high frequency of the CMT1A duplication in CMT patients, the availability of a quick and reliable method for detecting the 17p11.2 duplication will be very helpful for diagnosis, especially before clear manifestation of clinical symptoms.

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