Duplication within chromosome 17p11.2 in 12 families of French ancestry with Charcot-Marie-Tooth disease type 1a

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Abstract

Hereditary motor and sensory neuropathy type I (HMSN I), also designated Charcot-Marie-Tooth disease type 1 (CMT1), is a peripheral neuropathy frequently inherited as an autosomal dominant trait, characterised by progressive distal muscular atrophy and sensory loss with markedly decreased nerve conduction velocity. A duplication within chromosome 17p11.2, cosegregating with the disease, has recently been reported in several CMT1a families. In order to estimate the frequency of this anomaly and determine the location of a duplication in this region, 12 CMT1 families were analysed with polymorphic DNA markers located within 17p11.2-12. Duplications were found in all families including loci *D17S61* (EW401), *D17S122* (VAW409R3a and RM11-GT), and *D17S125* (VAW412R3). The duplications were completely linked and associated with the disease (lod score of 20.77 at zero recombination). Screening for the RM11-GT microsatellite showed that most of the duplicated haplotypes were heterozygous, supporting the hypothesis that the duplication resulted from an unequal crossing over. There was no significant haplotype association within the duplicated region suggesting that the duplication resulted de novo as an independent event in each family. In one family, recombination within the duplicated region was observed, indicating that genetic instability in 17p11.2 might be related to a high recombination rate. Since most cases of CMT1a seem to result from this segmental trisomy, it can be used as a basis for DNA diagnosis of the disease.

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The hereditary motor and sensory neuropathies (HMSN) represent a genetically heterogeneous group of diseases characterised by progressive muscular atrophy and sensory loss of the distal extremities. The most common of

the seven types described by Dyck¹ is HMSN I, also designated Charcot-Marie-Tooth disease type 1 (CMT1). In Europe, the prevalence is approximately 10/100 000.^{2,3} In the majority of cases, inheritance is autosomal dominant, although recessive, X linked, and sporadic cases have been reported.^{4,5}

Clinical onset usually occurs in the second or third decade.⁶ Patients present slowly progressive distal muscle weakness and atrophy, sensory loss, and absence of deep tendon reflexes, often associated with pes cavus.⁶ Clinical expression is extremely variable: some affected subjects remain asymptomatic their whole life, while others are confined to wheelchairs. All gene carriers, symptomatic or not, show markedly reduced nerve conduction velocities⁷ which is the basis for diagnosis. The diagnosis can be confirmed by nerve biopsies, where numerous onion bulb formations associated with extensive de- and remyelination are observed.¹

In spite of extensive clinical, electrophysiological, and neuropathological characterisation, the molecular basis of CMT1 has not been fully elucidated. Linkage studies represent the first step towards identification of the gene(s) responsible. Early studies suggested linkage to the Duffy blood group on chromosome 1,^{8,9} defining the CMT1b type, but this has not been confirmed in the majority of families.^{10,11} Positive linkage to markers on chromosome 17 was found later in most families.¹²⁻¹⁸ Further studies have restricted location of this form, designated CMT1a, to 17p11.2-p12.¹⁹⁻²¹ Recently, a DNA duplication, cosegregating with the disease, has been reported in several CMT1a families.^{22,23} We have studied 12 CMT1 families of French ancestry and have found a duplication in all affected subjects. The duplication spans at least three loci (*D17S61*, *D17S122*, *D17S125*) in the 17p11.2-p12 region in all pedigrees. The results suggest that the segmental trisomy resulting from this duplication could explain most cases of CMT1a.

Material and methods

FAMILIES

Twelve families of French ancestry, meeting strict selection criteria for CMT1, were analysed (fig 1). All at risk subjects underwent clinical and electrophysiological examination by the same neurologist (MG) to confirm CMT1 and exclude other types of peripheral neuropathy. The selection criteria, accepted

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Figure 1 Pedigrees of the 12 CMT1 families studied. Affected subjects are represented in black. Median motor conduction velocities are indicated below the subjects examined. * = subjects from whom blood samples were obtained.

Table 1 Mean median nerve conduction velocity in ms^{-1} (SD) in affected and unaffected at risk subjects in the 12 CMT1 families.

Family	Mean nerve conduction velocity			
	Affected (SD)	No	Unaffected at risk (SD)	No
254	17.7 (1.7)	3	64.0	1
256	23.4 (5.8)	7	58.2 (7.3)	16
260	19.1 (0.1)	3	54.0 (5.0)	4
261	20.4 (5.5)	5	58.6 (1.8)	4
263	19.6 (4.7)	8	59.0 (4.6)	6
264	24.0	1	—	—
272	24.9 (6.0)	9	57.1 (1.2)	6
274	17.5 (2.0)	5	51.0	1
281	25.0 (4.0)	2	—	—
283	21.0	1	—	—
284	22.5 (2.1)	2	57.7	1
285	21.3 (5.8)	3	—	—
Total	21.4 (5.4)	49	57.7 (5.7)	39

by the European HMSN Collaborative Research Group, for the probands were the following: (1) presence of slowly progressive symmetrical wasting and weakness in lower or upper limbs or both, distal sensory loss in lower or upper extremities or both, and areflexia at least at the ankles; (2) electromyographic signs of neuropathy, median motor nerve conduction velocity <30 m/s without conduction block, and absence of marked decrease in sensory action potentials in the lower limbs. For other at risk subjects, the presence of one of the clinical signs and a median nerve conduction velocity <30 m/s, or between 30 and 40 m/s in the two median nerves, were necessary for inclusion in the affected group. One hundred and two subjects, including 49 affected and 39 at risk persons, were examined. Mean median motor nerve conduction velocities are given for each family in table 1. All pedigrees showed dominant transmission, which was male to male in seven families, excluding X linked inheritance. Linkage with the Duffy blood group had previously been excluded in five families (261, 263, 272, 274, and 285). None of the remaining families had been previously analysed for linkage to chromosome 1 or 17 markers. Eight of these were informative and were used for linkage analysis (table 2).

Blood samples were obtained from all 102 subjects who gave their informed consent. High molecular weight DNA was purified according to a simplified procedure.²⁴ Permanent lymphoblastoid cell lines were established by transformation with the Epstein-Barr virus.²⁵

MARKER DETECTION

Two methods of genomic analysis were used: restriction fragment length polymorphism (RFLP) detection with DNA probes on Southern blots, and microsatellite analysis by PCR.

PROBES

DNA probes which recognise only single copy fragments were used. The inserts from probes EW401 (*D17S61*), VAW409R3a (*D17S122*), and VAW412R3 (*D17S125*) were kindly provided by C Van Broeckhoven (University of Antwerp, Antwerpen, Belgium). Probe VAW409R3a detects three *MspI* alleles with sizes of 2.8, 2.7, and 1.9 kb. Probes EW401 and VAW412R3 detect two *MspI* alleles with sizes of 10.5, 5.4 and 5.5, 4.4 kb, respectively. The inserts were labelled with (α -³²P) dCTP (Amersham) by the random priming method of Feinberg and Vogelstein.²⁶ A polymorphic (GT)_n marker, microsatellite RM11-GT, derived from pVAW409R1 and located at *D17S122*, has been characterised by Lupski *et al.*²³ This marker presents eight different alleles.

SOUTHERN BLOTS

Samples (8 μg) of genomic DNA were digested with the *MspI* restriction endonuclease according to the manufacturer's specifications (Bethesda Research Laboratories), in the presence of 4 $\mu\text{mol/l}$ spermidine. After verification of the digestion on a 0.5 μg aliquot, the digested DNA fragments were separated by electrophoresis on a 0.7% agarose gel in $1 \times$ TBE buffer (0.09 mol/l Tris-borate, 0.002 mol/l EDTA). The DNA was denatured by soaking the gels for 30 minutes in 0.5 mol/l NaOH, 1.5 mol/l NaCl, neutralised in 0.5 mol/l Tris-HCl (pH 7.5), 1.5 mol/l NaCl, 0.001 mol/l EDTA, and transferred overnight by capillary action to nylon membranes (Hybond N⁺, Amersham) in $10 \times$ SSC (1.5 mol/l sodium chloride, 0.15 mol/l sodium citrate, pH 7.0). After transfer, the DNA was fixed in 0.4 mol/l NaOH for 20 minutes then rinsed in $2 \times$ SSC. Filters were hybridised overnight at 65°C, according to Church and Gilbert,²⁷ washed in $0.1 \times$ SSC containing 0.1% SDS, at 65°C for 30 minutes, and exposed for autoradiography (Kodak X OMAT) with intensifying screens for one to three days at -70°C .

PCR AMPLIFICATION AT LOCUS *D17S122*

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) repeat. The 25 μl reaction contained 0.1 μg genomic DNA, 0.8 $\mu\text{mol/l}$ each oligodeoxynucleotide primer, one of which was 5' end labelled with (γ -³²P) ATP (Amersham), 200 $\mu\text{mol/l}$ each deoxynucleotide, 1 U of AmpliTaq DNA polymerase

Table 2 Lod scores for the duplication in eight informative CMT1 families.

Family	Recombination fraction						
	0.00	0.01	0.05	0.10	0.20	0.30	0.40
254	0.60	0.59	0.54	0.47	0.32	0.17	0.05
256	6.93	6.81	6.37	5.78	4.51	3.08	1.50
260	1.81	1.77	1.63	1.44	1.04	0.62	0.24
261	2.41	2.37	2.23	2.04	1.63	1.17	0.63
263	3.61	3.55	3.30	2.97	2.26	1.45	0.57
272	3.91	3.85	3.60	3.27	2.56	1.76	0.89
274	1.20	1.18	1.09	0.98	0.73	0.46	0.21
284	0.30	0.29	0.26	0.21	0.13	0.06	0.02
Total	20.77	20.41	19.02	17.16	13.18	8.77	4.11

(Cetus) in $1 \times$ PCR buffer (0.05 mol/l KCl, 0.0015 mol/l $MgCl_2$, 0.01 mol/l Tris-HCl pH 8.3, 0.01% gelatin). After an initial denaturation for three minutes at $94^\circ C$, 29 cycles of amplification with denaturation for 30 seconds at $94^\circ C$, annealing for 30 seconds at $55^\circ C$, and extension for 30 seconds at $72^\circ C$, followed by a 10 minute final extension at $72^\circ C$, were performed in an automated thermal cycler (Perkin Elmer/Cetus). Reaction products were denatured at $90^\circ C$ for three minutes, loaded in a 30% formamide dye solution on an 8% denaturing polyacrylamide gel, and run at 40 W for four hours. Gels were dried by heating under vacuum and autoradiographed for one to two days (Kodak X OMAT x ray films).

LINKAGE ANALYSIS

Two point lod scores for linkage of the duplication with CMT1 were calculated for the eight informative families using the program MLINK from LINKAGE version 5.1.²⁸ The duplication was scored for each subject as if it were a biallelic system (present or absent). CMT1 was considered to be a fully penetrant autosomal dominant trait. Frequencies of the CMT1 gene and duplication of the 17p11.2 region were considered to be 0.0001. Male and female recombination rates were assumed to be equal.

Figure 2 Duplication at locus D17S122 in 12 families. The lanes represent an affected subject from each family (fig 1) and one control. Top: autoradiograph of an *MspI* Southern blot hybridised with the VAW409R3a probe. Bottom: autoradiograph of the PCR products obtained with RM11-GT. 1, 2, and 3 correspond to *MspI* alleles of 2.8, 2.7, and 1.9 kb, respectively. 1 to 7 represent the RM11-GT alleles. The genotypes and the duplicated haplotypes (separated by a slash), which were determined by segregation analysis, are indicated below the autoradiographs. Figures are presented in parentheses when the duplicated haplotype could not be determined.

Results

In order to estimate the frequency of duplication in these families, we analysed all the sampled subjects with probe VAW409R3a (D17S122) and with the microsatellite RM11-GT located within the same region in 17p11.2-p12.²³ The duplication appeared as either three different alleles, or two different alleles, one of which was present in two copies giving a stronger hybridisation signal. The autoradiograph in fig 2 illustrates the duplication observed with probes VAW409R3a and RM11-GT in one patient from each family. The duplication, as visualised with probe VAW409R3a, consisted of the three different alleles in two patients (256 II-2, 261 III-4) and a stronger hybridisation signal for one of two alleles in eight affected heterozygotes (260 II-2, 263 II-10, 264 I-2, 272 IV-2, 274 I-1, 283 III-1, 284 III-2, and 285 II-2). In the two affected subjects who were homozygous with probe VAW409R3a, the duplication could be visualised with the RM11-GT microsatellite as three different alleles (254 II-2) or different dosages of two alleles (281 III-1). Forty-nine affected patients, but none of the 39 unaffected at risk subjects or 24 spouses, had the duplication. Segregation of the duplication with the disease was observed in the eight informative families where meiosis had occurred without recombination. The maximal lod score for linkage of the duplication with CMT1 was 20.77 at $\theta = 0.00$ cM (table 2).

Two other loci in the region, D17S61 and D17S125, were explored with probes EW401 and VAW412R3. All affected subjects carried duplications at these loci as well. An example of the genotypes of the members of family 261 obtained with the four probes is given in fig 3.

In most cases, the duplicated alleles were heterozygous: six were discriminated with VAW409R3a and eight with RM11-GT in nine families where the haplotypes could be determined. Four different haplotypes for probe VAW409R3a were observed in the nine families. There was no significant difference ($\chi^2 = 8.69$, $0.10 > p > 0.05$) between the expected number of haplotypes and those observed (table 3). With the highly polymorphic RM11-GT microsatellite, seven out of nine duplicated haplotypes were different (fig 2).

In one subject (family 256, III-14), recombination in the duplicated region was observed with the microsatellite RM11-GT, characterised by a change of the duplicated haplotype from 6-6 to 3-6 (fig 4). This event must have occurred during meiosis in subject II-5.

Discussion

This study shows the presence of a duplication within chromosome 17p11.2-p12 in 12 unrelated families with CMT1. Stringent diagnostic criteria (median nerve conduction velocity < 30 m/s for the index case) allowed rigorous selection of the families. A clear cut difference in mean median nerve conduction velocity was observed between the affected and unaffected at risk groups. Electrophysiological

Figure 3 Duplication of loci D17S61, D17S122, and D17S125 in members of family 261. (A) Partial pedigree showing subjects studied. (B) Genotypes obtained by PCR with the marker RM11-GT. RM11-GT alleles are indicated by the numbers 2 to 6. (C, D, E) MspI alleles visualised on Southern blots hybridised with probes VAW409R3a, VAW412R3, and EW401, respectively. Numbers 1, 2, and 3 of VAW409R3a correspond to the MspI alleles 2.8, 2.7, and 1.9 kb, respectively. Numbers 1 and 2 of VAW412R3 and EW401 correspond to the MspI alleles 10.5, 5.4 and 5.5, 4.4 kb, respectively.

Table 3 Haplotypes of MspI alleles detected with probe VAW409R3a in nine CMT1 families.

Haplotype	Haplotype frequency	Expected No of haplotypes	Observed No of haplotypes
1,1	0.250	2.250	0
1,2	0.440	3.960	3
1,3	0.060	0.540	1
2,2	0.193	1.737	3
2,3	0.053	0.477	2
3,3	0.004	0.036	0
Total	1	9	9

Figure 4 Recombination at locus D17S122 in family 256. Genotypes and duplicated haplotypes (separated by a slash) obtained by PCR with marker RM11-GT are indicated below each subject.

examination of all at risk subjects permitted identification of 16 asymptomatic gene carriers, who were included as affected in the study. Without this procedure almost one-third of the affected patients would not have been identified. All the affected subjects of these multigeneration families had the duplication, which segregated with the disease without recombination, as reflected by the maximal lod score of 20.77 at $\theta = 0.00$. Raeymaekers *et al*²² and Lupski *et al*²³ have already reported 20 families with duplications in the affected patients. It seems likely that this partial trisomy will be present in most, if not all, autosomal dominant CMT1 families.

It is probable that the duplication is responsible for the CMT1 phenotype since it segregates specifically with the disease and was not found in any of the unaffected at risk subjects or spouses. The simultaneous appearance of the disease with a *de novo* duplication²² is conclusive evidence of its pathogenicity. Thus, CMT1a is the first autosomal dominant human disease where a microduplication can be held responsible for most of the cases.

The exact size of the duplication has not yet been determined. In our 12 families it spans three 17p11.2-p12 loci (D17S61, D17S122, and D17S125). No junction fragments were observed on Southern blots with any of the three probes (EW401, VAW409R3a, or VAW412R3). The probes hybridise with a single *NotI* fragment of approximately 1.1 Mb,^{29,30} which is, thus, the minimal size of the duplicated region. The mechanism by which the duplication induces the CMT1a phenotype is unclear, however. Overexpression of a duplicated gene (gene dosage effect) is a possible explanation, considering that more than 10 genes can be found in the duplicated region. Another possibility would be the interruption of a gene at the duplicated junction. This hypothesis can be confirmed by breakpoint analysis in several families.

The mechanism by which the duplication is generated is also unknown. The high frequency of heterozygosity within the duplication for locus D17S122 indicates that it could result from unequal crossing over. The results of Raeymaekers *et al*²² also support this hypothesis. The lack of significant haplotype association within the duplicated region for probe VAW409R3a is in favour of the notion

that the duplication results from an independent de novo event within each family. The finding obtained with RM11-GT microsatellite, that eight out of nine duplicated haplotypes are different, reinforces this hypothesis. On the other hand, the haplotype of an ancestral duplication could have been altered by frequent recombination in 17p11.2. This possibility should be considered, as Wright *et al*³¹ determined the genetic distance between loci *D17S61* and *D17S122* to be 13.9 and 4.2 cM in females and males, respectively, values which are surprisingly high considering that the physical distance does not exceed 1.1 Mb. The discrepancy between a small physical and a large genetic distance suggests that meiotic recombination occurs often in the 17p11.2 region. The observation of a recombination in family 256 with microsatellite RM11-GT is compatible with this assertion. Whether the other loci were involved in the recombination or not could not be determined, as no blood sample from subject II-6 was available. Raeymaekers *et al*³⁰ also reported two recombinations within the duplicated region. Estimation of the frequency of de novo duplications will require analysis of families with sporadic cases before a definite conclusion can be drawn.

The presence of a microduplication in autosomal dominant CMT1a provides an easy method for the DNA diagnosis of CMT1a in familial or sporadic cases, obviating the need for large families required by linkage analysis with chromosome 1 or 17 markers. The frequency of homozygotes, which renders the duplication difficult to show, can be markedly reduced by the use of several different probes. Most of the patients had two or three different alleles with the very informative RM11-GT microsatellite, a marker which permits direct demonstration of the duplication. However, interpretation of genotypes with the RM11-GT microsatellite may be difficult if only two different alleles are present. The use of RFLP probes in conjunction with RM11-GT microsatellite would permit the most accurate diagnosis. Screening of patients with the duplication will allow the clinical and electrophysiological spectrum of the disease to be defined better.

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