European Journal of Human Genetics (2000) 8, 229–235

www.nature.com/ejhg

ARTICLE

Prenatal detection of a 17p11.2 duplication resulting from a rare recombination event and novel PCR-based strategy for molecular identification of Charcot-Marie-Tooth disease type 1A

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Charcot-Marie-Tooth disease, type 1A (CMT1A) is caused in most cases by a 1.5 Mb duplication on chromosome 17p 11.2 arising after unequal crossing-over between repeated sequences called *CMT1A-REP*s, flanking the 1.5 Mb unit. A 3.2 kb recombination hot spot has been defined, resulting in a junction fragment between *Eco*RI (distal *CMT1A-REP*) and *Sac*I (proximal *CMT1A-REP*). This was further reduced to a 1.7 kb *Eco*RI-*Nsi*I fragment, and recently to a 731 bp hot spot region within this fragment. We describe the CMT1A-REPs-based PCR method used to identify CMT1A duplications and report on a family case in which a 29-year-old pregnant woman requested prenatal diagnosis for two successive pregnancies because her husband was affected with CMT1A. Our method enabled us to characterise the duplication in both foetuses and demonstrate that it arose from a rare recombination event taking place outside the 1.7 kb region. Since our approach is simple and enables the entire set of duplications occurring after recombination in the enlarged 3.2 kb region including the hot spot to be detected, we suggest it might be considered for use in primary screening for pre- and postnatal diagnosis of CMT1A. *European Journal of Human Genetics* (2000) **8**, 229–235.

Keywords: Charcot-Marie-Tooth; CMT1A; *CMT1A-REP*s; recombination; prenatal diagnosis; PCR; genetic counselling

Introduction

Charcot-Marie-Tooth (CMT) disease is a hereditary motor and sensory neuropathy which encompasses a heterogeneous group of disorders of which CMT type 1 (CMT1), the demyelinating or hypertrophic form, is the most common.[1]

Several loci have been identified for CMT1.[2] CMT1A, the most common subtype, is linked to chromosome 17.[3] The abnormally lowered NCVs observed in CMT1A patients directly correlate with the stable inheritance of a 1.5 Mb duplication on chromosome 17p11.2–p12.[4] This duplicated region contains a gene coding for the peripheral myelin protein 22 (*PMP22*), which is the dosage-sensitive gene involved in the CMT1A phenotype.[5–9] However, in rare instances, point mutations occur in the *PMP22* gene in CMT1 patients without duplication.[10,11]

It has been postulated that the duplicated chromosome in CMT1A results from a homologous recombination event occurring in meiosis between misaligned repeat sequences flanking the 1.5 Mb region (proximal and distal *CMT1A-REP*s).[12] Two groups have demonstrated that the recombination event usually occurs within a 3.2 kb *Eco*RI–*Sac*I interval

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Received 10 September 1999; revised 19 November 1999; accepted 23 November 1999

between the misaligned distal and proximal *CMT1A-REP*s.[13,14] This interval was reduced to a 1.7 kb *Eco*RI–*Nsi*I fragment and strategies have been developed to detect a junction fragment by Southern blot analysis.[13–15] This fragment is specific for the CMT1A duplication and is present in 75–80% of CMT1A duplicated patients. Recently, we have refined the recombination hot spot to a 741 bp interval in this region.[16]

The methods currently used in most laboratories for molecular diagnosis of CMT1A (Southern blots, microsatellite analysis) are time consuming and are usually based on isotopically labelled probes. Moreover, they are not always reliable (eg dosage analysis), or informative (eg polymorphic analysis). Alternatively, both pulse field gel electrophoresis (PFGE) to detect a junction fragment and fluorescent *in situ* hybridisation (FISH) to determine the *PMP22* copy number have been employed. PFGE analysis, one of the most reliable methods, widely performed in the US,[17] is technically challenging, requiring special equipment and laboratory expertise. Polymerase chain reaction (PCR) based strategies have been designed to detect CMT1A recombinations. They make it possible to detect a 1.7 kb junction fragment, but miss the entire set of duplications in the 3.2 kb region.[18,19] More recently, Stronach *et al*,[20] reported a PCR assay which allows both CMT1A and HNPP recombination events occurring within the hot spot to be detected. They obtained 100% correlations after comparison between usual and PCR-based procedures to detect CMT1A duplications in the hot spot.

In this report we describe a novel methodology based on a PCR assay, which identifies the most common molecular rearrangement involved in CMT1 disease. Moreover, we used a panel of microsatellites included in the CMT1A duplicated region to identify duplications which cannot yet be identified by CMT1A-REPs PCR assay since they occur after recombination outside the 3.2 kb region. This procedure, designed as a quick, economical, simplified method for CMT1A duplication detection, was successfully employed in two cases of prenatal diagnosis for CMT1A, requested by the same couple for two consecutive pregnancies. It allowed identification of the CMT1A duplication arising from a very rare recombination event outside the 1.7 kb region. Since this approach detects the whole set of duplications occurring after recombination within the 3.2 kb region, it might be widely used for pre- and postnatal diagnosis of CMT1A disease.

Materials and methods

Subjects and samples

CMT1A duplicated patients and normal controls previously explored for the CMT1A locus were included in the analysis. The parents requesting prenatal diagnosis were non-consanguineous individuals, the father was affected with CMT1A, as was his mother, and several other members of his family (Figure 1). Genomic DNAs were extracted from blood lymphocytes under conditions according to Maniatis.[21] DNA from foetuses (III4 and III5) was phenol extracted after trophoblastic biopsy at 14 weeks of pregnancy.

CMT1A-REPs based PCR assay

A set of primers encompassing the recombination hot spot involved in the generation of the duplication was selected in regions of maximal divergence between the proximal and distal *CMT1A-REP*s (GenBank HSU41165 and HSU41166).[13] The forward primer was designed for the distal *CMT1A-REP* downstream from the *Eco*RI site (*Rdist1*) and the reverse for the proximal *CMT1A-REP* upstream of the *Sac*I site (*Rprox2*) (Figure 2). These primers amplify a 3.7 kb fragment.

Primer sequences were as follows:

Rdist1: 5' GGATTCAGAGACATTAGTGTTAC 3' (base 1500 to 1523 of sequence HSU41165)

Rprox2: 5' GGTTCCATGAGGAAACCAGAATT 3' (base 5177 to 5154 of sequence HSU41166)

PCR reactions were carried out under the following conditions, 100 ng of genomic DNA was amplified using *Taq* (Roche, Meylan, France) according to the manufacturer's instructions in a Hybaid thermocycler for 30 cycles (denaturation: 30 s at 92°C, annealing: 45 s at 60°C, and elongation: 4 min at 68°C), with an initial denaturation at 92°C for 2 min and a final elongation at 68°C for 10 min. Amplified products were then digested by *Eco*RI + *Nsi*I (5U each/PCR

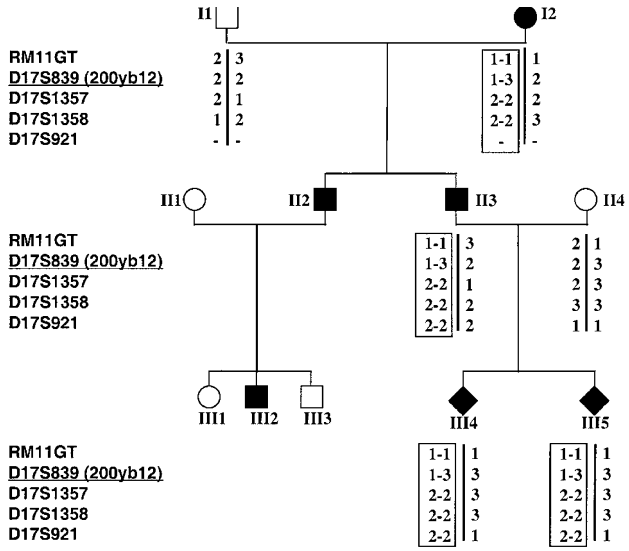

Figure 1 Pedigree of the CMT1A family. Haplotypes are indicated below individual symbols. Marker D17S839 (200yb12) is underlined as it indicates three separated alleles in the affected members and allows us to follow the transmission of the duplicated chromosome.

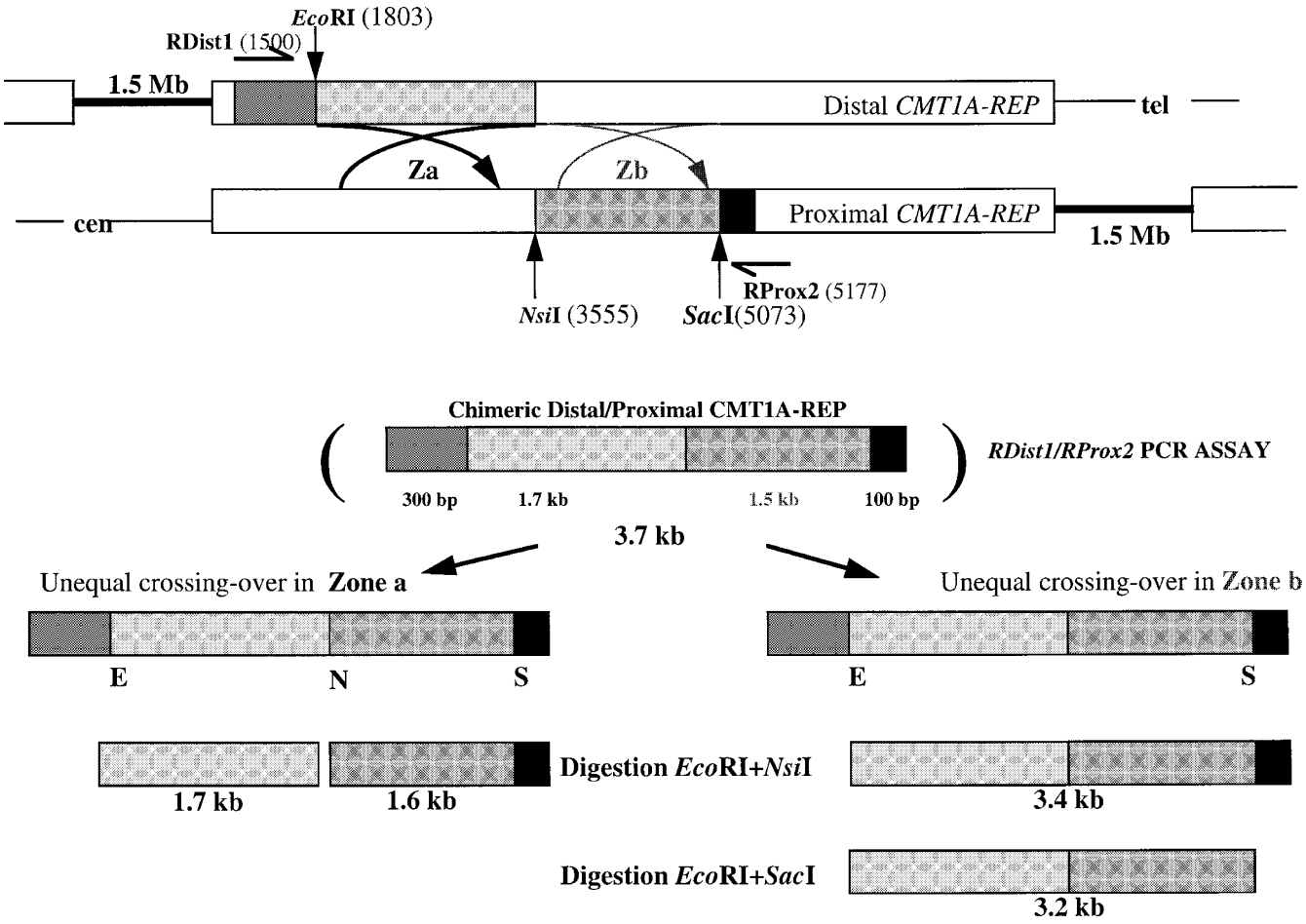

Figure 2 *Schematic* representation of the *CMT1A-REP*s PCR-based strategy and recombination events. *Eco*RI + *Nsi*I digestion indicates a 1.7 kb (junction fragment) and a 1.5 kb (*Nsi*I-*RProx2* fragment) in patients with recombination in the 'hot spot'. *Eco*RI + *Sac*I digestion only provides a 3.2 kb junction fragment in this family because of absence of the *Nsi*I site on the recombinated CMT1A-REP element. Za: 1.755 kb recombination zone a, containing the hot spot; Zb: 1.52 kb recombination zone b, corresponding to the rare event described in the family. *RDist1*: forward primer designed to the distal CMT1A-REP element; *RProx2*: reverse primer designed to the proximal CMT1A-REP element. E: *Eco*RI; N: *Nsi*I; S: *Sac*I. For both primers and restriction sites, positions are indicated by numbers in parentheses corresponding to the distal and proximal CMT1A-REPs sequences (Genbank accession numbers: HSU41165 and HSU41165 respectively).

reaction for 2 h) and/or *Eco*RI + *Sac*I. Samples were electrophoresed on an 0.8% ethidium bromide stained agarose gel.

Microsatellite analysis

PCR reactions for five microsatellites included in the CMT1A monomer unit (duplicated region), RM11GT, D17S839 (AFM200yb12), D17S1357, D17S1358, D17S921 (AFM181 × h12), were performed under standard conditions modified as follows. For each microsatellite, forward primer was 5' labelled (fam), while reverse primer was unlabelled; 25 mg of each primer were added to the PCR mixture. Samples were loaded in polymer pop4 on an ABI310 and results were interpreted with Genescan analysis software (PE Biosystems, Countaboeuf, France).

Results

CMT1A-REPs targeted PCR assay

Both recombination events explored by *CMT1A-REP*s targeted PCR assay and anticipated results are summarised in Figure 2.

Under the conditions described in the materials and methods section, a single band at 3.7 kb was obtained for normal and CMT1A individuals. After subsequent digestion by *Eco*RI/*Nsi*I, a 1.7 kb junction fragment was identified for CMT1A controls carrying a duplication caused by recombination in the hot spot. A 1.6 kb fragment, originating from the *Nsi*I-*Rprox*2 fragment of the recombinant *CMT1A-REP* was also observed in the same patients (Figure 3a). Moreover, a 3.4 kb and a 300 bp band corresponding to *Eco*RI digestion of the fragment amplified from the distal REP were also found

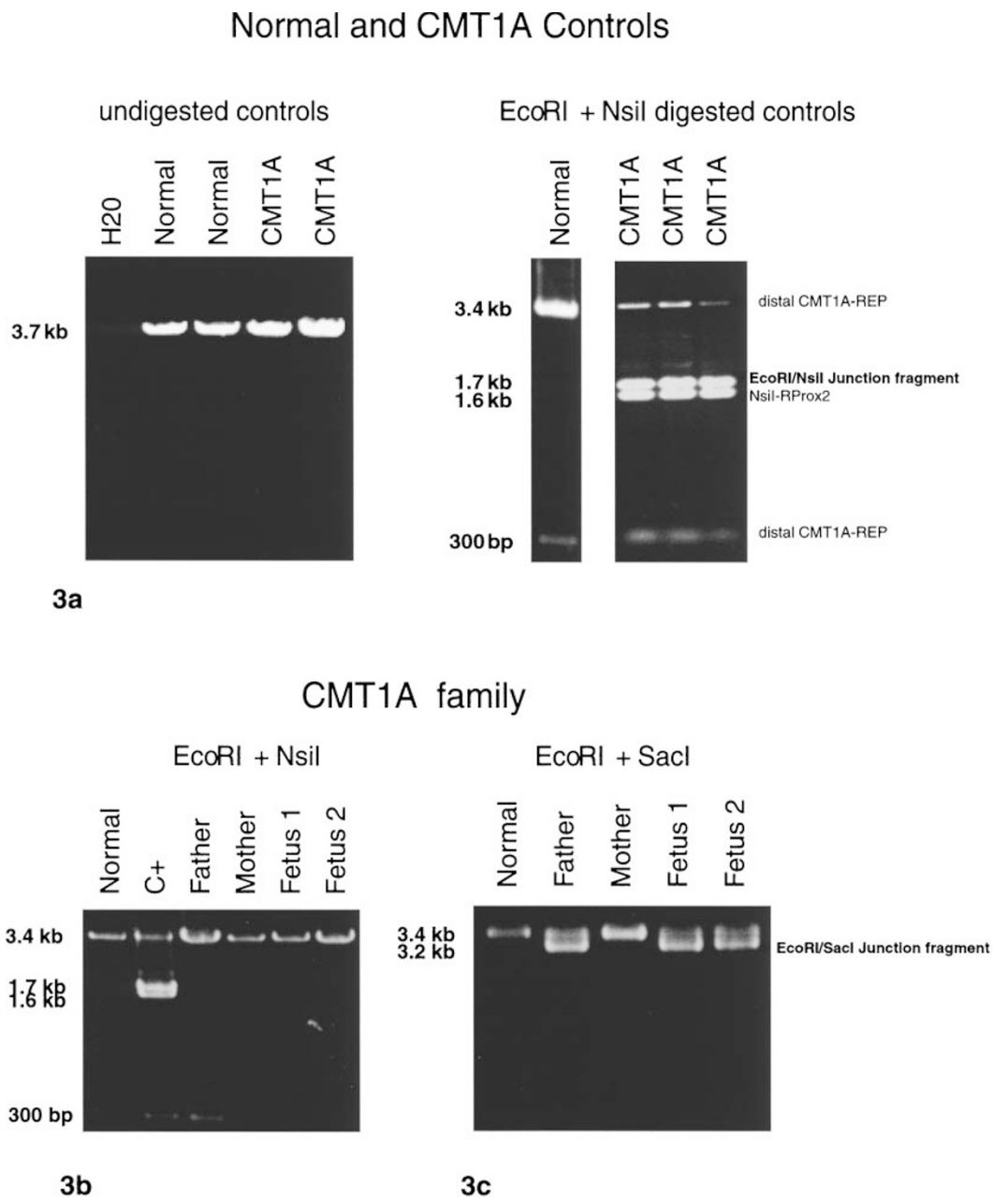

Figure 3 CMT1A-REPs targeted PCR results. **a** Normal and CMT1A controls DNAs were amplified and loaded on agarose gel before (left) and after *Eco*RI + *Nsi*I digestion (right). Fragments at 3.7 kb are obtained for all the individuals (normal and CMT1A) before digestion; fragments at 3.4 and 300 bp correspond to the normal distal CMT1A-REPs and fragments at 1.7 and 1.6 kb correspond to the recombinated chimeric CMT1A-REP after recombination within the hot spot. **b** In the family reported here, no hot spot specific fragments were visualised after *Eco*RI + *Nsi*I digestion and only fragments at 3.4 kb and 300 bp were present, corresponding to both distal and recombinated *CMT1A-REPs*. C+, CMT1A positive control. **c** *Eco*RI + *Sac*I digestion of the PCR products showed a 3.2 kb junction fragment in the father and foetuses, which was absent from the mother and normal controls.

in all individuals (patients and controls) (Figure 3). Because the primers were designed in diverse regions between distal and proximal REPs, the proximal *CMT1A-REP* was never amplified or cut.

Surprisingly, no 1.7 kb and 1.6 kb bands were visualised in the father although he harbours an *Eco*RI–*Sac*I 3.2 kb junction fragment detected by Southern blot (data not shown). Those hot spot specific fragments were not found in the foetuses, the mother and negative controls (Figure 3b). We therefore performed an *Eco*RI/*Sac*I digestion on the same PCR products. This resulted in a 3.2 kb band in samples from the father and the foetuses (Figure 3c). Since, in CMT1A, the

chimeric *CMT1A-REP* is the only one that contains both *Eco*RI and *Sac*I restriction sites, we concluded the foetuses had inherited the duplication from their father. No such 3.2 kb band was obtained for the mother and negative controls. This rearrangement is due, in this family, to a rare recombination event that occurred in a 1.5 kb region upstream of the *Nsi*I site (*Nsi*I–*Sac*I fragment). These results were confirmed by Southern blot analysis evidencing the duplication in affected members and foetuses by gene dosage analysis[7] and the presence of a 3.2 kb junction fragment[14] (data not shown).

Microsatellite analysis

We used a panel of five polymorphic microsatellites included in the 1.5 Mb monomer unit,[22] which when combined gave a high probability of detecting three unique alleles in duplicated individuals.

For D17S839 (200yb12), three alleles of different sizes were found, indicating the presence of the duplication in the grandmother (I2), transmitted to the father (II3), and inherited by the foetuses (III4 and III5) (Figure 4). Genotyping results are also given in the pedigree in Figure 1.

Discussion

The reliability of our approach was tested initially in a preliminary study performed in 50 CMT1A duplicated individuals (R Bernard-Bronsard, 1997, personal communication). A large series of CMT1A patients already diagnosed by gene dosage analysis, and/or after detection of a 3.2 kb junction fragment by Southern blot analysis, together with normal individuals as negative controls, was studied this way. The results of the Southern blot and PCR analysis were identical, indicating that this *CMT1A-REP* targeted PCR approach detects 100% of duplications resulting from a recombination in the 3.2 kb region including the recombination hot spot.[23]

We therefore propose a screening strategy to detect the entire set of duplications involved in CMT1A. This strategy is based on first screening potentially duplicated individuals (using clinical, genealogical, and electrophysiological data), by the *CMT1A-REP*s targeted PCR assay reported in this paper. This approach represents a novel PCR-based strategy compared with the previously reported PCR assays using *CMT1A-REP* specific primers for the detection of the CMT1A duplication.[18–20,24] Such a strategy provides a new tool for the diagnosis of hereditary peripheral neuropathies which is rapid, cost-effective, reproducible, and without biohazards. If the initial screening using the *CMT1A-REP*s targeted PCR assay is not informative (absence of either the 1.7 kb *Eco*R1–*Nsi*I or the 3.2 kb *Eco*RI–*Sac*I junction fragments), we suggest the use of a polymorphic microsatellite panel of markers included in the 1.5 Mb monomer unit.[22] Indeed, patients in whom a specific junction fragment is not identified represent 20% of our patient base. This microsatellite analysis alone

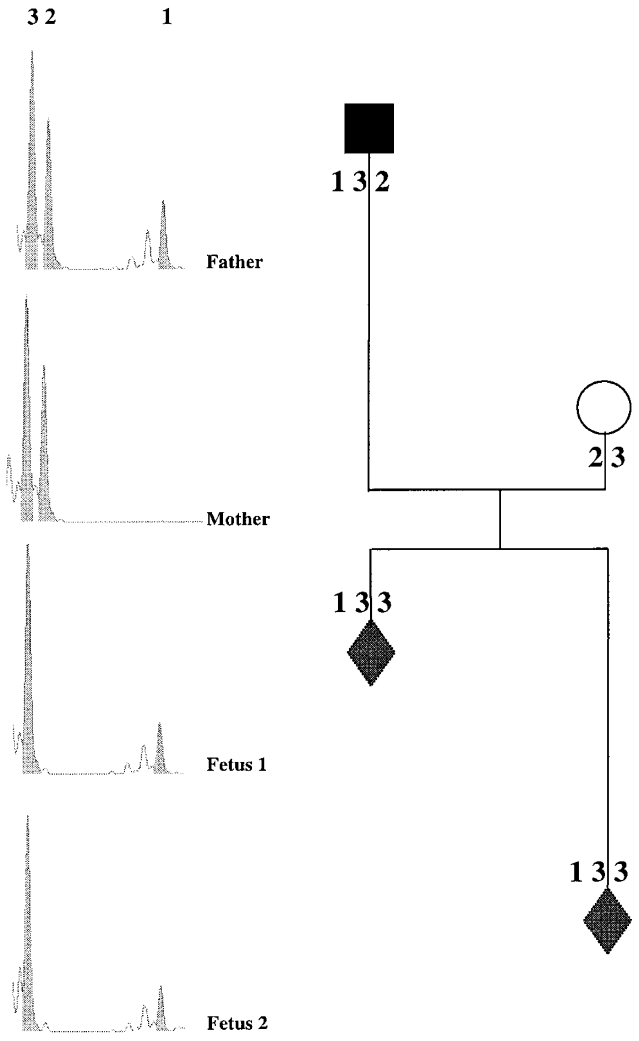

Figure 4 Results of microsatellite D17S839 (200yb12). Results of microsatellite D17S839 on ABI 310 automated sequencer (Genescan software) identifying three separate alleles from the father and the transmission of the duplicated chromosome to both foetuses (see Figure 1 for genotyping results). For each individual, the corresponding alleles are indicated by numbers at the top of the figure.

was validated by Blair *et al*[25] who detected 85% of patients carrying the 1.5 Mb duplication. In our experience, this combined strategy detects all the duplications involved in familial CMT1A. Gene dosage analysis by Southern blot should be carried out only in the very rare remaining non-informative cases, especially sporadic cases, for whom a recombination event lies outside the 3.2 kb region. In such cases, FISH analysis may also be an interesting alternative.[26,27]

With regard to prenatal diagnosis, the advantage of using a technique based only on a single PCR assay followed by a

short digestion is evident, primarily because of the rapidity of the method. Indeed, an interval of 6 days from sampling (trophoblastic biopsy) to result can be considered very short.

To date, very few cases of prenatal diagnosis of CMT, have been reported.[26,28,29] Herein, we first report the prenatal detection of a CMT1A duplication by use of a *CMT1A-REP*s PCR based strategy.

The duplication carried by the father and the two foetuses was verified by most of the usual technical procedures used for CMT1A diagnosis (microsatellite analysis and Southern blot). We show that the *Rdist1/Rprox2* PCR assay, followed by an *Eco*R1 + *Sac*I digestion allows the detection of a 3.2 kb junction fragment in the father's and the foetuses' DNA, which is absent in the mother's as well as in unaffected individuals' DNA. Thus, all the tests performed in this family using four independent approaches demonstrate complete coherence of result and the reliability of our strategy for identification of duplications in CMT1A. Moreover, in this particular family, our results indicate that the exchange event took place in a very rare recombination zone corresponding to a 1.5 kb sequence, between the *Nsi*I site (virtual site on the distal *CMT1A-REP*) and the *Sac*I site on the proximal *CMT1A-REP* (see Figure 2).

A possible major concern is ethic-related, due to the difficulty of genetic counselling and the opportunity for prenatal diagnosis for both non-lethal and clinically very heterogeneous diseases, such as CMT1A, even within the same family.

The family explored in our study was a typical example of such clinical variability; although the father was slightly affected as was his mother, his nephew (III2) had a very severe form of the disease, incompatible with normal life. Although the karyotype was normal in the first foetus explored, the parents decided to terminate the pregnancy because of a possible severe phenotype associated with the CMT1A duplication. After an 8 month interval, the parents again requested a prenatal diagnosis for a second pregnancy. The foetus was examined by the same procedures and we concluded he was also carrying the duplication inherited from his father. The parents decided to terminate the pregnancy. The relative merits of preimplantation diagnosis, not yet feasible or available, and early prenatal diagnosis, to limit psychological effects due to pregnancy termination should be discussed, in diseases with unpredictable severity of the phenotype and high risk of recurrency. However, although one case of preimplantation genetic diagnosis in CMT1A has been reported,[30] success by this approach is case dependent, requiring perfect technical conditions and further large scale evaluation. In addition, we strongly believe that efficient and patient genetic counselling in CMT1A should lead to few families requesting prenatal diagnosis. Based on these comments, early prenatal diagnosis seems to be the most appropriate approach, particularly since the sampling to result turnaround time may be reduced to less than a week by using PCR strategy such as described in this report.

Acknowledgements

We thank Michel Fontes, Michael J Mitchell for helpful discussions and proof-reading the manuscript, Caroline Piquet for performing the chromosomal analysis on fetuses and Valery Ravix for technical assistance.

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