

Charcot-Marie-Tooth disease type 1A (CMT1A) and hereditary neuropathy with liability to pressure palsies (HNPP): Reliable detection of the CMT1A duplication and HNPP deletion using 8 microsatellite markers in 2 multiplex PCRs

PAVEL SEEMAN¹, RADIM MAZANEC², JANEZ ZIDAR³, SÁRKA HRUSÁKOVÁ¹,
MARKÉTA CTVRTECKOVÁ¹ and BERND RAUTENSTRAUSS⁴

Departments of ¹Child Neurology, and ²Neurology, 2nd School of Medicine, Charles University Prague, V úvalu 84, 15006 Praha 5, Czech Republic; ³University Institute of Clinical Neurophysiology, Medical Centre, Zaloska 7, 61105 Ljubljana, Slovenia; ⁴Department of Human Genetics, University Erlangen-Nürnberg, Schwabachanlage 10, 91054 Erlangen, Germany

Received June 19, 2000; Accepted July 18, 2000

Abstract. Charcot-Marie-Tooth disease (CMT) and hereditary neuropathy with liability to pressure palsies (HNPP) are the most frequent inherited disorders of the peripheral nervous system. They are clinically and genetically heterogeneous. A submicroscopic tandem duplication of 1.5 Mb in chromosome 17p11.2-12 comprising the PMP22 gene is found in 70.7% of autosomal dominant Charcot-Marie-Tooth type 1 (CMT1) patients. A reciprocal deletion is found in 87.6% of HNPP patients. The size of the typical CMT1A duplication is too small for classical cytogenetics and the whole region including the CMT1A-REP elements is sometimes too complex for a single DNA analysis method. We present results of a multiplex PCR of 8 microsatellite markers with multicolour fluorescence primer labelling followed by fragment analysis on an ABI 310 Prism analyzer to simplify the diagnostic procedure. Results for 24 patients can be obtained within 24 h. This method was applied on 92 DNA samples of unrelated patients carrying a typical CMT1A duplication previously confirmed by two colour fluorescence *in situ* hybridization (FISH, probe c132G8) and *EcoRI/SacI* Southern blotting (probe pLR7.8). Three alleles of three different sizes were clearly detected at least once in 88 of them (95.6%). Subsequently this analysis was applied on 312 Czech patients and revealed a CMT1A/HNPP rearrangement in 109 out of them.

Introduction

Charcot-Marie-Tooth disease type 1 (CMT 1) and tomaculous neuropathy, also called hereditary neuropathy with liability to pressure palsies (HNPP), are subgroups of hereditary motor and sensory neuropathies (HMSN) (1).

CMT 1 is a common, autosomal dominantly inherited disorder of the peripheral nervous system clinically characterised by distal muscle weakness and wasting, foot deformities (pes cavus), absent deep tendon reflexes and an age of onset typically in the late first or early second decade (1).

Electrophysiologically a severely decreased nerve conduction velocity (NCV <38 m/sec) due to demyelination of peripheral nerves is present (1).

The vast majority of the CMT1 patients carry a large, but submicroscopic duplication in chr. 17p.11.2.-12 (CMT1A), where the gene for peripheral myeline protein 22 (PMP22) is located (2,3). Rarely, point mutations in PMP22 have also been reported (4). Also, mutations in the Myelin Protein Zero (CMT1B) gene, the Connexin 32 (CMTX) and EGR2 gene have been described as causing CMT type 1 (7).

HNPP is usually a milder peripheral nerve disorder clinically characterised by recurrent palsies on different extremities, following insignificant trauma or slight pressure on the peripheral nerve (5).

Decreased NCV is sometimes found in electrophysiological studies (4). The pattern of inheritance is also autosomal dominant (4). A deletion in 17p.11.2.-12 of the CMT1A region can be found in the vast majority of HNPP patients (6).

Various detection methods for these rearrangements are used by different laboratories. Pulsed-field gel electrophoresis (PFGE), quantitative Southern blotting, fluorescence *in situ* hybridisation (FISH) and various PCR methods, including microsatellites, are the most common and most powerful of them (7,8).

Eight polymorphic microsatellites, dinucleotide markers, were previously proven to be located within this duplicated/deleted region in 17p.11.2.-12 (9-11). These 8 markers

Correspondence to: Dr Pavel Seeman, Department of Child Neurology, 2nd School of Medicine, Charles University Prague, V úvalu 84, 150 06 Praha 5, Czech Republic
E-mail: pavel.seeman@lfmotol.cuni.cz

Key words: Charcot-Marie-Tooth type 1, hereditary neuropathy with liability to pressure palsies, hereditary motor and sensory neuropathies, microsatellite markers, multiplex PCR, fragment analysis

Table I. Microsatellite (CA)_n markers used in set 1 and set 2.^a

Set 1 at 58°C			
AFM 200	(D17S839)	GDB 189384	[Tet]
RM11GT	(D17S122)	GDB 186801	[Fam]
AFM 191	(D17S921)	GDB 199142	[Fam]
133C4	(D17S1358)	GDB 439442	[Hex]
Set 2 at 55°C			
142E8	(D17S1356)	GDB 439299	[Fam]
103B11	(D17S1357)	GDB 439405	[Fam]
AFM 317	(D17S955)	GDB 200333	[Hex]
Mfd 41	(D17S261)	GDB 177322	[Tet]

^aThree synonyms are used for every marker. GDB represents genome database number (www.gdb.org). Tet, Fam and Hex represent the dye for fluorescein labelling of forward primers.

promised to be well suited for a quick and reliable detection of rearrangements of the CMT1A region.

We used fluorescence labelled primers for PCR amplification of these previously described dinucleotide repeat regions. The resulting fluorescence labelled PCR products are then analysed on an automated Genetic Analyzer ABI 310 (Perkin-Elmer, USA). Assuming the presence of a CMT1A duplication there should be a very high probability to find three alleles of different sizes in one or more of the markers used. In the case of an HNPP deletion, affected persons do not show two alleles in any of the markers used and in a segregation analysis the lack of allelic transmission from an affected parent to an affected offspring is always found.

We report our experience with this rapid and inexpensive detection method for the two most common mutations in inherited peripheral neuropathies.

Patients and methods

Patients were either referred from the Neurological Department of the Friedrich-Alexander University, Erlangen, Germany, from the Departments of Child and Adult Neurology at the Charles-University in Prague, Czech Republic or the Neurological Department at the Medical Center in Ljubljana, Slovenia. The 92 CMT1A duplication carriers preselected by fluorescence *in situ* hybridization (FISH) and *EcoRI/SacI* Southern blot suffered from distal weakness, muscle atrophy and sensory disturbances. Most of these patients have at least one affected relative. These patients had markedly reduced nerve conduction velocities below 38 m/sec measured from the median, ulnar and tibial nerves as sign of a demyelinating neuropathy. Some of the patients underwent a sural nerve biopsy and morphological evaluation showing signs of a hypertrophic demyelinating neuropathy. The genetic study by the microsatellite multiplex

PCR method was extended to 312 patients with a wider phenotypic variability referred from Czech neurologists also beyond the Charles University. For these patients the clinical data were not available for the authors hence also other PNP are probably included. For those who did not show a CMT1A duplication the point mutation screening in the genes for PMP22, Cx32, MPZ and EGR2 is ongoing.

Genomic DNA was isolated from peripheral lymphocytes according to standard protocols. Eight dinucleotide (CA)_n microsatellite markers (Table I) were amplified in two multiplex PCR reactions under the following thermoprofile: 95°C for 5 min, then 25 cycles of 95°C for 1 min, 58°C or 55°C for 1 min, 72°C for 1.5 min, final extension 10 min at 72°C in a thermocycler PTC 100 (MJ Research, USA). Forward primers of each marker are labelled with a fluorescein dye. Markers where the sizes of the possible alleles can overlap are labelled with different colours. Markers RM11GT, labelled with Fam (Perkin-Elmer, USA), AFM 191, labelled with Fam, AFM 200 labeled with Tet (Perkin-Elmer, USA) and 133C4 labelled with Hex (Perkin-Elmer, USA) are amplified in the set 1 at 58°C. Markers 142E8 labelled with Fam, AFM 317 labelled with Hex, 103B11 labelled with Fam and Mfd 41 labelled with Tet are amplified in the set 2 at 55°C (Table I). The total reaction volume is 15 µl for each system. Thin wall 0.2 ml sample tubes were used, the content of PCR mixtures is shown in Table II.

PCR was followed by control agarose gel electrophoresis using 5 µl of PCR product. About 1 µl of PCR product was then mixed with 12 µl of deionised formamide and 0.4 µl fluorescent size standard TAMRA 500 (Perkin-Elmer, USA). This mixture was directly loaded in the Genetic Analyzer ABI 310 (Perkin-Elmer, USA) without denaturation (Table III). Usual injection time is 6 sec and electrophoresis for 20 min is sufficient. After capillary electrophoresis in a POP 4 polymer (Perkin-Elmer, USA) the number, size and intensity ratio of resulting PCR products (alleles) in each marker were analyzed using Gene Scan 3.1 software (Perkin-Elmer, USA).

Results

We developed and tested a microsatellite multiplex PCR based detection method for the CMT1A duplication and HNPP deletion.

At first 92 DNA samples derived from CMT patients previously tested to carry a CMT1A duplication proven by two independent routine tests (FISH, probe c132G8; *EcoRI/SacI* Southern hybridization, probe pLR7.8) were analysed by this multiplex PCR method. Finding of three different alleles was estimated for each marker and mainly for combination of all 8 markers used (Fig. 1).

Three alleles of different sizes in at least one of the marker used were detected in 88 out of the 92 DNA samples (Fig. 1) which represents 95.6% (Fig. 2). In the other four DNA samples an allele dosage difference was visible indicating also the typical CMT1A duplication. The most informative marker was RM11GT with 46.6% of samples showing three alleles. Using only the four most informative markers, namely RM11GT, AFM 191, 133C4, 103B11, the detection rate of three alleles was still 88%. According to these results the detection of three alleles in at least one of the markers

Table II. Multiplex PCR mixture protocols for primer sets 1 and 2.

Set 1 at 58°C									
DNA 200 ng/μl	10X PCR buff.	dNTP 10 mM each	Taq 1 U/μl	Primer mix 10 pmol/μl each (forward + reverse)				MgCl ₂ 25 mM	H ₂ O
				RM11GT Fam	191 Fam	200 Tet	133 Hex		
0.5 μl	1.5 μl	0.6 μl	0.6 μl	2.4 μl	0.6 μl	0.5 μl	1.2 μl	1.2 μl	6 μl
Set 2 at 55°C									
DNA 200 ng/μl	10X PCR buff.	dNTP 10 mM each	Taq 1 U/μl	Primer mix 10 pmol/μl each (forward + reverse)				MgCl ₂ 25 mM	H ₂ O
				142 Fam	317 Hex	103 Fam	Mfd 41 Tet		
0.5 μl	1.5 μl	0.6 μl	0.6 μl	1.2 μl	1.2 μl	1.2 μl	0.8 μl	1.2 μl	6.2 μl

Table III. Protocol of the CMT1A duplication/HNPP deletion detection method with approximate time periods for each step.

1. DNA isolation from peripheral lymphocytes	2 h
2. 2 multiplex PCRs each with 4 pairs of primers labelled with different fluorescein dyes	2.5 h
3. Control agarose gel electrophoresis	0.5 h
4. ~1 μl from PCR product (unpurified) + 12 μl formamid + 0.4 μl size standard TAMRA 500	1 h/48 spl
5. Capillary electrophoresis on ABI 310	20 h/48 spl
6. Fragment number and fragment length analysis with GeneScan 3.1 program	2 h/48 spl
Total for 24 patients	28 h

together with a dosage difference in another marker confirms the typical CMT1A duplication (Fig. 3).

Now this method is further used as a routine DNA test for patients with suspicion for inherited peripheral neuropathies in Czech Republic. We have used this method already in 312 of these CMT/HNPP patients from 122 families. We detected the CMT1A duplication in 82 of these patients (Table IV), but three alleles were not detectable in any of the 8 markers used for four of the patients. However, there was a clear allele intensity ratio difference in all four of these non-three-allele patients. All these four patients without three alleles had severely decreased NCV and their offspring carry a CMT1A duplication proven by three microsatellite alleles or

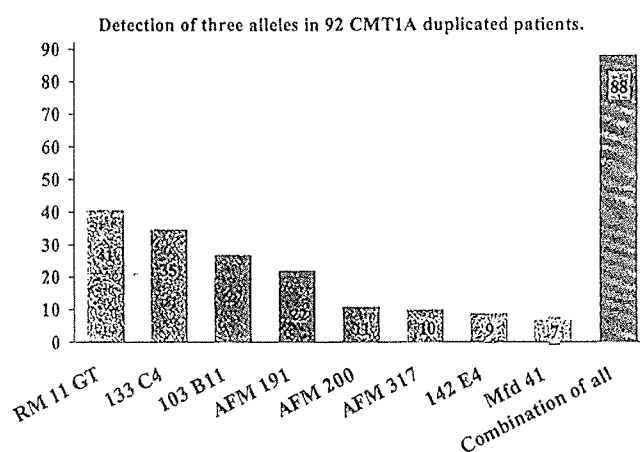


Figure 1. Numbers of DNA samples for each marker used where three alleles of different length were detected. The 'combination of all' column represents the number of DNA samples where three different alleles were detected in at least one of the 8 markers used.

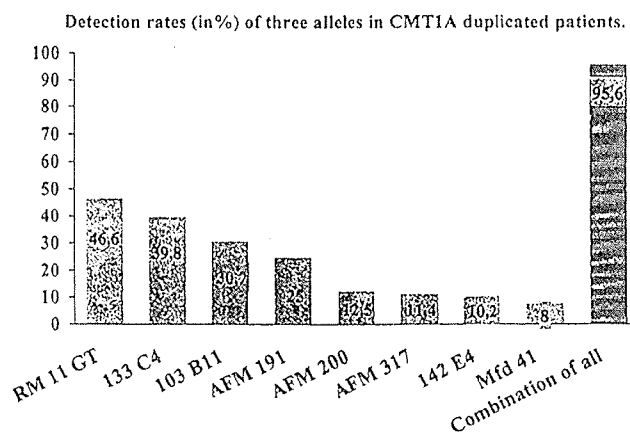


Figure 2. Percentage of DNA samples for each marker used. 'Combination of all' means here the percentage of DNA samples where 3 alleles of different size where detected in at least one of the 8 markers used.

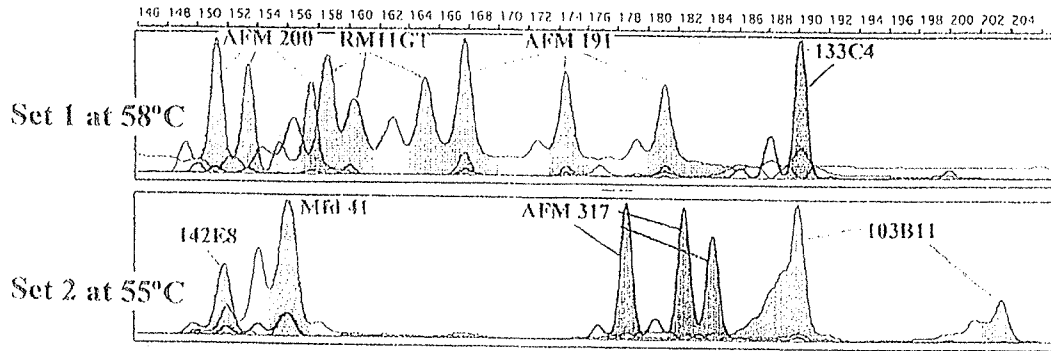


Figure 3. Example for a result from one CMT1 patient carrying the CMT1A duplication. Upper line, primer set 1; lower line, primer set 2. Note the presence of three alleles of different size in markers AFM 200, RM11GT, AFM 191 and AFM 317 and the clearly visible intensity difference between both alleles in marker 103B11, which results from the presence of two alleles of the same length (190 bp). Numbers on the top line represent the sizes of alleles in basepairs (bp).

CMT1A specific FISH. These four samples account for 4.8% and seem to be in agreement with the results from our test with 92 confirmed duplicated samples, where also four samples had not displayed three alleles of different size.

All other samples showed clear allele intensity ratio differences compatible with a CMT1A duplication in the investigated region.

Finding one allele only in all 8 markers used is highly suggestive for the HNPP deletion (Fig. 4). The lack of an allele transmission from the affected parent to the affected offspring confirms the presence of the HNPP deletion in both generations (Fig. 4). By these criteria we identified 27 individual HNPP deletion carriers from 15 families.

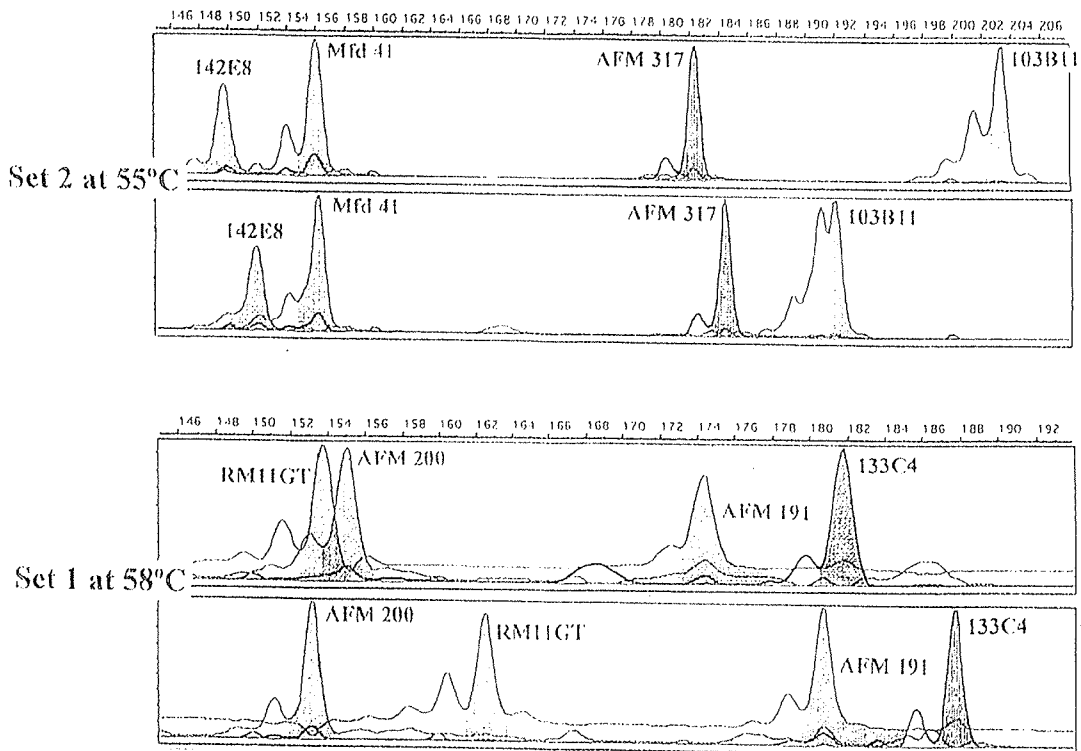


Figure 4. Results from a female HNPP patient (lower lines) and her affected son (upper lines). Note the presence of only one allele (PCR product) in all 8 markers used and the lack of allele transmission from the affected mother to the affected offspring in markers AFM 200, RM11GT, AFM 191, 133C4, 142E8, AFM 317 and 103B11. The chr. 17p11.2 alleles of the son were not transmitted from his mother, hence they are of paternal origin. Data of the father were not available. Numbers along the top lines show the sizes of alleles in basepairs (bp), respectively.

Table IV. Summarized results of the analysed CMT/HNPP patients in Czech Republic.

Total number of investigated patients	312
Total number of investigated families	122
CMT1A duplication families	37
CMT1A duplication individuals	82
HNPP deletion families	15
HNPP deletion individuals	27

In summary we detected a chr. 17p11.2 rearrangement in 52 out of 122 Czech index patients indicating a frequency of 43% (Table IV).

Discussion

We developed and tested a PCR based method for reliable detection of the most common mutations in inherited peripheral neuropathies.

This method is very simple, rapid and cost effective and allows a high throughput of samples. It is therefore well suitable for a large number of samples as well as for a small clinical laboratory. However, alone it does not allow a complete exclusion of a duplication for the rare cases with only increased dosage of marker alleles, in our two analyses this occurred in about 4% of the cases. Hence it can reliably confirm the presence of a CMT1A duplication for the vast majority of carriers. The cases in doubt should then be checked by other methods like Southern hybridisation or FISH (15-18). Our results on 8 microsatellite markers allow an increase in the detected number of CMT1A duplications compared to data reported by Blair *et al* (9) using 4 microsatellite markers, i.e. the total proportion of samples that exhibited three alleles of different size in at least one of the markers is increased. Furthermore, our study included a more representative number of confirmed CMT1A duplications. In comparison we used nearly twice as many DNA samples and 4 more markers in total. We showed that our method is able to detect three alleles in 95.6% of proven CMT1A duplication samples.

By the combination of the 4 most informative markers, RM11GT, AFM 191, 133C4, 103B11, three alleles in at least one of these markers were detectable in 88% of the proven duplicated samples. However, this method does not allow the full exclusion of a CMT1A duplication. This holds especially true for CMT1A duplications that arose not from unequal crossing over but sister chromatid recombination (14).

In order to try to further increase the detection rate of three alleles we analysed a more stable tetranucleotide marker, namely D17S71 located in chr. 17p12, in 6 Czech HNPP patients from 3 families and 5 unrelated CMT patients. These patients were confirmed to be mutation carriers by the microsatellite method. However, the HNPP patients showed two alleles of different length and a lack of allele transmission

was not found in any of 3 investigated HNPP families. Neither three alleles nor dosage difference between the two alleles were found in any of the 5 CMT1A patients. Hence this marker is probably located outside the CMT1A region and not suitable for the duplication/deletion screening.

The use of 2 multiplex PCR with four markers in each reaction and the multicolour labelling results in great time, labour and cost savings. The method has also a potential for high throughput of many samples and allows us to have results from 24 patients in one or two days. The detection rate of 95.6% for three alleles is comparable to other routinely used single probe Southern blotting methods. By the use of quantitative evaluation of the PCR products, which is commonly used for example in PCR based detection of chromosomal aneuploidies, it is possible to detect almost all duplicated samples. The microsatellite markers used for our PCR based assay are distributed across the 1.5 Mb CMT1A region, thereby maximizing the probability of one or more being included within an atypical shortened duplication. Our optimisation of known single methods can provide a powerful tool for laboratories with a high throughput as well as small clinical laboratories interested in peripheral neuropathies.

Acknowledgments

P.S. is supported by a grant of IGA MH CR Nr. M/3-3 and by Investigation Project No 111300003 to 2nd School of Medicine Prague. This work was also funded by DFG and DGM to B.R.

References

- Dyck PJ, Chance P, Lebo R and Carney JA: Hereditary motor and sensory neuropathies. In: *Peripheral Neuropathy*, Vol. 2, 3rd edition. Dyck PJ (ed). WB Saunders, Philadelphia, pp1094-1132, 1993.
- Nelis E, van Broeckhoven C, De Jonghe P, *et al*: Estimation of the mutation frequencies in Charcot-Marie-Tooth disease type 1 and hereditary neuropathy with liability to pressure palsies: an european collaborative study. *Eur J Hum Genet* 4: 25-33, 1996.
- Lupski JR, Montes de Oca-Luna R, Slaugenhaupt S, *et al*: DNA duplication associated with Charcot-Marie-Tooth disease type 1A. *Cell* 66: 219-232, 1991.
- De Jonghe P, Timmerman V, FitzPatrick D, Nelis E, Martin J-J and van Broeckhoven C: Charcot-Marie-Tooth disease and related peripheral neuropathies. *J Periph Nerv Syst* 2: 370-387, 1997.
- De Jong JG: About families with a hereditary disposition to compression of the nerves correlated with migraine. *Psychiatr Neurol BI* 50: 60-76, 1947.
- Chance PF and Fischbeck KH: Molecular genetics of Charcot-Marie-Tooth disease and related disorders. *Hum Mol Genet* 3: 1503-1507, 1994.
- Pareyson D: Charcot-Marie-Tooth disease and related neuropathies: molecular basis for distinction and diagnosis. *Muscle Nerve* 22: 1498-1509, 1999.
- Lupski JR: DNA diagnostics for Charcot-Marie-Tooth disease and related inherited neuropathies. *Clin Chem* 42: 995-998, 1996.
- Blair IP, Kennerson ML and Nicholson GA: Detection of Charcot-Marie-Tooth duplication by the polymerase chain reaction. *Clin Chem* 41: 1105-1108, 1995.
- Cudrey C, Chevillard C, Pashier D, Vignal A, Passage E and Fontes M: Assignment of microsatellite sequences to the region duplicated in CMT1A (17p12): a useful tool for diagnosis. *J Med Genet* 32: 231-233, 1995.

11. Le Guern E, Ravise A, Goudier R, Gugenheim M, Lopes J, Bouche P, Agid Y and Brice A: Microsatellite mapping of the deletion in patients with hereditary neuropathy with liability to pressure palsies (HNPP): new molecular tools for the study of the region 17p12-p11 and for diagnosis. *Cytogenet Cell Genet* 72: 20-25, 1996.
12. Rautenstrauss B, Liehr T, Fuchs C. *et al.*: Mosaicism for Charcot-Marie-Tooth disease type 1A: onset in childhood suggests somatic reversion in early developmental stages. *Int J Mol Med* 1: 333-337, 1998.
13. Reiter LT, Murakami T, Koeuth T. *et al.*: A recombination hotspot responsible for two inherited peripheral neuropathies is located near a mariner transposon/like element. *Nat Genet* 12: 288-297, 1996.
14. Lopes J, Ravise N, Vandenberghe A, Palau F, Ionasescu V, Mayer M, Levy N, Wood N, Tachi N, Bouche P, Latour P, Ruberg M and Brice A: Fine mapping of *de novo* CMT1A and HNPP rearrangements within CMT1A-REPs evidences two distinct sex-dependent mechanisms and candidate sequences involved in recombination. *Hum Mol Genet* 7: 141-148, 1998.
15. Bettecken T, Schneider V, Burgunder J-M and Moser H: Molekulargenetische Diagnostik der Hereditären Motor-Sensorischen Neuropathien (HMSN) und der Familiären Rekurrierenden Neuropathie (HNPP). *Med Genetik* 6: 20-21, 1994.
16. Timmerman V, Rautenstrauss B, Reiter LT, *et al.*: Detection of the CMT1A/HNPP recombination hotspot in unrelated patients of European descent. *J Med Genet* 34: 43-49, 1997.
17. Lier T, Thoma K, Gehring C. *et al.*: Direct preparation of uncultured EDTA-treated or heparinized blood for interphase FISH analysis. *Appl Cytogenet* 21: 185-188, 1995.
18. Roa BB, Ananth U, Garcia CA, *et al.*: Molecular diagnosis of CMT1A and HNPP Charcot-Marie-Tooth disease type 1A and hereditary neuropathy with liability to pressure palsy. *Lab Med Int* 12: 22-24, 1995.