ORIGINAL ARTICLE

Allele-specific All-or-None PCR Product Diagnostic Strategy for Charcot-Marie-Tooth 1A and Hereditary Neuropathy with Liability to Pressure Palsies

Kon-Ping Lin[1,2]*, Chi-Hsiang Chou[2,3], Hsiang-Ying Lee[1], Bing-Wen Soong[1,2]

[1]The Neurological Institute, Taipei Veterans General Hospital, Taipei, [2]Department of Neurology, National Yang-Ming University School of Medicine, Taipei, and [3]Department of Neurology, Yuan-Shan Veterans General Hospital, I-Lan, Taiwan, R.O.C.

Background: We designed allele-specific primers to amplify genomic DNA of patients with Charcot-Marie-Tooth 1A (CMT1A) and hereditary neuropathy with liability to pressure palsies (HNPP).

Methods: Genomic DNA analysis was performed on 40 unrelated CMT1A duplication patients, 25 unrelated HNPP deletion patients, and 50 unaffected control individuals. The CMT1A and HNPP patients had previously been identified with microsatellite mapping.

Results: Amplification products came to 3.6 kb in length from the normal proximal CMT1A repeated segment on chromosome 17p11.2 (proximal CMT1A-REP), 3.57 kb from the normal distal CMT1A repeated segment on chromosome 17p11.2 (distal CMT1A-REP), 3.6 kb from HNPP patients, and 3.58 kb from CMT1A patients. We could identify the mutations by means of agarose gel electrophoresis after polymerase chain reaction (PCR) amplification without restriction enzyme digestion from 33 of the 40 CMT1A and 19 of the 25 HNPP samples.

Conclusion: Stringently specific primers were used to overcome the problem of nonspecific amplification and provide a rapid, all-or-none PCR product and efficient screening test for CMT1A and HNPP. [*J Chin Med Assoc* 2006;69(2): 68–73]

Key Words: allele-specific primers, Charcot-Marie-Tooth disease, hereditary neuropathy with liability to pressure palsy

Introduction

Charcot-Marie-Tooth (CMT) disease is the most frequent inherited peripheral neuropathy, with an estimated prevalence of approximately 1 in 2,500.[1] Two major forms of CMT can be identified electrophysiologically: one shows defects in the formation or maintenance of myelin (CMT1) and the other refers to primary axonal degeneration (CMT2).[2] A microduplication of 1.5 Mb containing the gene for peripheral myelin protein 22 (PMP22) on chromosome 17p11.2 is responsible for 75% of cases of the demyelinating form (CMT1A).[3–9] Diagnosis with markers located inside the duplication is usually carried out using restriction fragment length polymorphism (RFLP) probes[3,4,10] and poly(AC) repeats,[11,12] less frequently with fluorescence *in situ* hybridization (FISH) analysis,[13] rapid real-time fluorescent polymerase chain reaction (PCR) gene dosage,[14] and multiplex ligation-dependent probe amplification (MLPA).[15] Molecular diagnosis often relies on the interpretation of differences in allele intensities, even with the most polymorphic markers that have been reported to date. When poly(AC) repeats are used for molecular diagnosis, artifact bands produced by slippage of the polymerase enzyme may lead to difficulties in interpretation of dosage for different alleles. Other useful diagnostic methods have been

*Correspondence to: Dr. Kon-Ping Lin, Neurological Institute, Taipei Veterans General Hospital, 201, Section 2, Shih-Pai Road, Taipei 112, Taiwan, R.O.C.
E-mail: kplin@vghtpe.gov.tw • Received: March 14, 2005 • Accepted: September 8, 2005

developed after extensive investigation of the 2 repeated 24-kb sequences (REPs) flanking the duplicated region.[16] A strategy with polymorphic short tandem repeats (STRs) located inside the CMT1A duplicated region that allows amplification with very low or no stuttering, accurate sizing, and visual quantification of allele intensity was used efficiently.[17]

Recombination events leading to the CMT1A duplication occur almost entirely in 4 adjacent regions within 7.8 kb of the CMT1A-REP sequences, with a "hot spot" of recombination located in a 3.2-kb central segment defined by 2 restriction enzyme sites, *Eco*RI and *Sac*I.[18,19] Characterization of the junction fragment resulting from recombination allows positive identification of the duplication with RFLP probes on Southern blots.[18,19]

Several methods with direct allele-specific amplification of the junction fragments by PCR have also been reported.[20–23] However, these methods were time-consuming and were used with restriction enzymes to define results. We developed an allele-specific, all-or-none and rapid PCR method to detect the junction fragments of CMT1A and hereditary neuropathy with liability to pressure palsies (HNPP).

Methods

Genomic DNA analysis was performed on 40 unrelated CMT1A duplication patients, 25 unrelated HNPP deletion patients, and 50 unaffected control individuals. The CMT1A and HNPP patients had previously been identified with microsatellite mapping: (ATCT)3AT(ATCT)6(ACCT)5 (GenBank accession no. AC005703), (CAATA)14 (GenBank accession no. AC0013248), (TTTC)12 (GenBank accession no. AC0013248), D17S122 (RM11GT), and D17S921(afm191xh12).[17,24] The CMT1A and HNPP PCR assays described here were carried out in a blind study. Informed consent was obtained from all subjects recruited in this study before genetic testing.

Genomic DNA was extracted from peripheral lymphocytes.[25] The following allele-specific primers were designed according to the published proximal and distal CMT1A-REP sequences (GeneBank accession no. U41165-distal and U41166-proximal): Hot-DF 5' TTGGATTCACAGAGACATTAGTGTTAC-3'; Hot-DR 5'-TAGTAGAGTGAGTACAGTGGAC-3'; Hot-PF 5'-TTGGATTCAAAGATATTAGTGTTAT-3'; Hot-PR 5'-TAGTAGAGCTCACTCTACAG-3'.

The primers Hot-DF and Hot-DR were used for the normal distal CMT1A-REP, the primers Hot-PF and Hot-PR were used for the normal proximal CMT1A-REP, the primers Hot-DF and Hot-PR were used for the junctional CMT1A repeated fragment, and Hot-PF and Hot-DR were used for the junctional HNPP repeated fragment.

Amplification was carried out in 30 μL of 1.5 mM $MgCl_2$, 50 pmol of each primer, 250 μmol of each dNTP, 50 ng template DNA, and 2.5 units *Taq* DNA polymerase (Takara Bio Inc., Shiga, Japan). The PCR buffer (10×) was composed of 100 mM Tris-HCl (pH 8.3), 500 mM KCl, and 15 mM $MgCl_2$. Amplification was done by initial denaturation at 94°C for 5 minutes followed by 25 cycles of 30 seconds at 94°C, 1 minute at 56°C, and 3 minutes at 72°C, including a 1-second autoextension function on the extension time with a final extension of 5 minutes at 72°C using a PTC-200 Peltier thermal cycler (MJ Research, Watertown, MA, USA). Amplified products were digested with *Eco*RI and *Nsi*I (New England Biolabs, Beverly, MA, USA) according to the manufacturer's instructions and electrophoresed in 1% agarose gels. Gels were stained in ethidium bromide (0.1 μg/mL) and visualized under UV light.

Results

The normal distal and proximal CMT1A-REP PCR assay amplified 3.57- and 3.6-kb segments, respectively, from 50 normal subjects, 40 CMT1A patients, and 25 HNPP patients using Hot-DF and Hot-DR or Hot-PF and Hot-PR primers. The CMT1A PCR assay amplified a 3.58-kb junctional CMT1A-REP fragment using primers Hot-DF and Hot-PR. However, there was no PCR product in 50 normal subjects and 25 HNPP patients using this pair of primers. In the HNPP PCR assay, a 3.6-kb junctional CMT1A-REP fragment was amplified using primers Hot-PF and Hot-DR. Furthermore, there was no PCR product in 50 normal subjects and 40 CMT1A patients (Figure 1).

Those PCR products of normal distal CMT1A-REP, normal proximal CMT1A-REP, junctional CMT1A, and HNPP fragments could be further digested with *Eco*RI and *Nsi*I to confirm their effective amplification. The normal distal CMT1A-REP segment could be digested by *Eco*RI, but not *Nsi*I, to generate 3.17- and 0.4-kb fragments. The normal proximal CMT-REP segment could be digested by *Nsi*I, but not *Eco*RI, to generate 2.1- and 1.5-kb fragments. The junctional CMT1A fragment could be digested by *Eco*RI and *Nsi*I to generate 1.7-, 1.5-, and 0.4-kb fragments. The junctional HNPP fragment could not be digested by *Eco*RI or *Nsi*I (Figure 2).

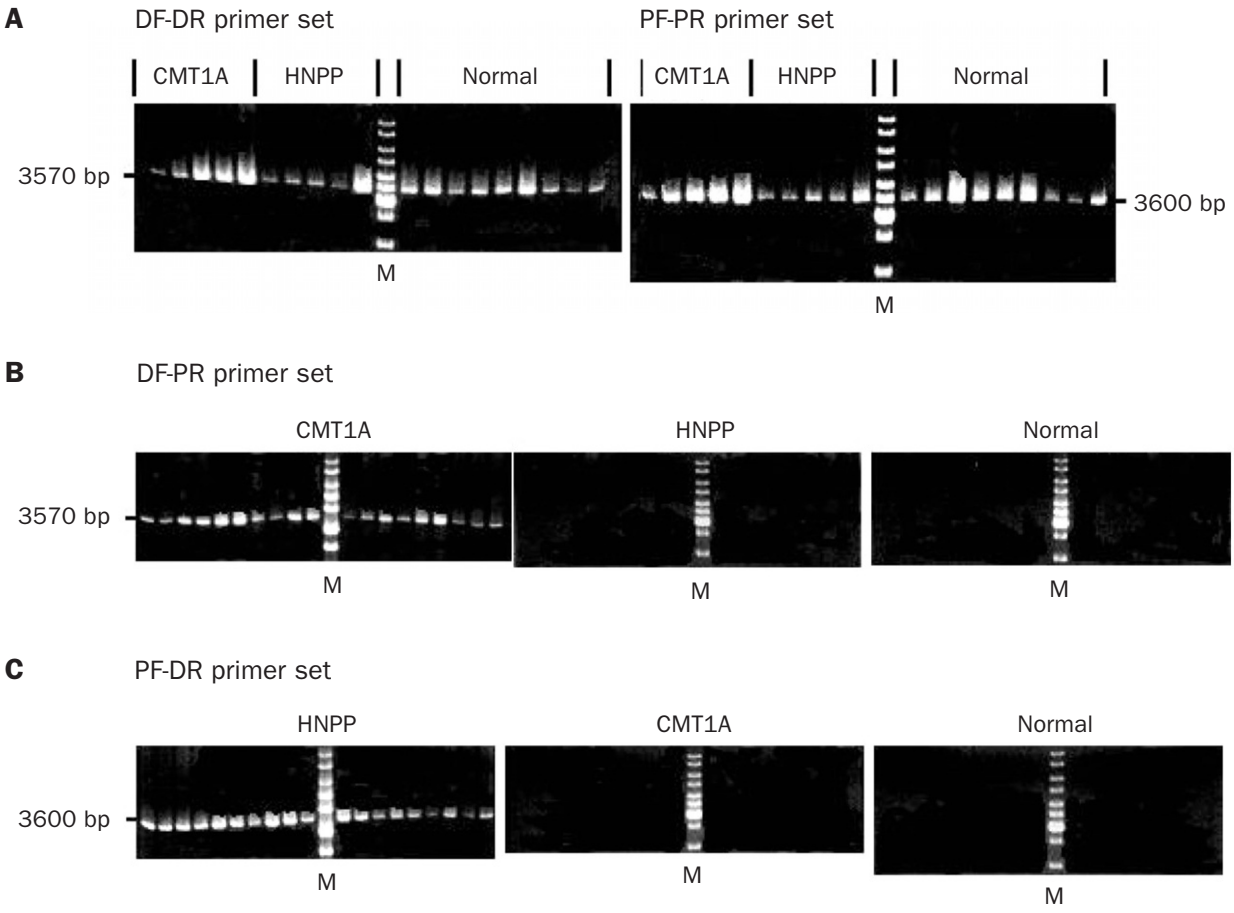

Figure 1. (A) DF-DR primer set can produce a 3.57-kb PCR product and PF-PR a 3.6 kb product in CMT1A, HNPP and normal controls. (B) DF-PR primer set can produce a 3.57-kb PCR product only in CMT1A, but not in HNPP and normal controls. (C) PF-DR primer set can produce a 3.6-kb PCR product only in HNPP, but not in CMT1A and normal controls. bp = base pair; M = base-paired marker.

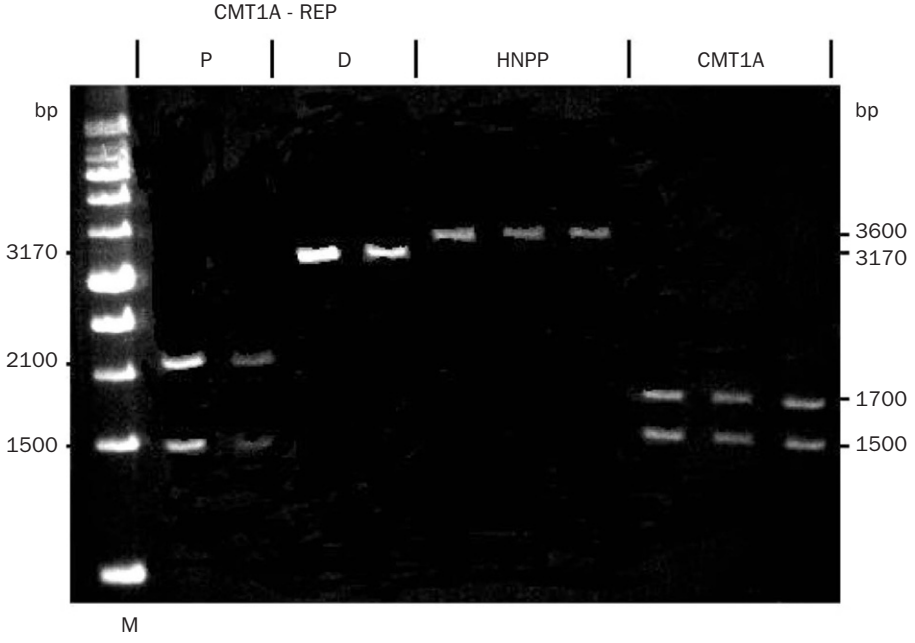

Figure 2. Restriction analysis of *Eco*RI and *Nsi*I in PCR products of proximal CMT1A-REP, distal CMT1A-REP, HNPP, and CMT1A with agarose gel electrophoresis and ethidium bromide staining. There were 2.1 and 1.5 kb in proximal CMT1A-REP (*Eco*RI restriction site), 3.17 and 0.4 kb (not shown) in distal CMT1A-REP (*Nsi*I restriction site), 3.6 kb in HNPP (no restriction sites of *Eco*RI and *Nsi*I in recombination fragments of HNPP) and 1.7, 1.5 and 0.4 kb (not shown) in CMT1A (*Eco*RI and *Nsi*I restriction sites). bp = base pair; M = base-paired marker; P = proximal; D = distal.

The CMT1A PCR test detected duplications in 33 of the 40 samples. As expected, the 33 positive results were obtained from individuals shown by microsatellite analysis to have crossover breakpoints within the 3.2-kb region, while the remaining 7 lay outside the 3.2-kb region. The HNPP PCR test detected deletions in 19 of the 25 samples. As expected, the 19 positive results were obtained from individuals shown by microsatellite analysis to have a crossover event within the 3.2-kb region, while the remaining 6 lay outside the 3.2-kb region. No false positives were detected in 50 unaffected controls tested with both primer sets.

Discussion

Using our method, we have detected 82.5% of CMT1A and 76% of HNPP patients in this study. Since the duplication of the *PMP-22* gene accounts for 70.7% of cases of CMT1 and deletion of the *PMP22* gene was present in 84% of the HNPP patients,[9,23] screening for *PMP22* gene duplications/deletions obviously should be the first approach in diagnosing hereditary demyelinating neuropathy.

Currently available techniques for detecting gene duplication or deletion are laborious and have specific drawbacks. Southern blot analysis is a labor-intensive technique involving the use of radioisotopes, chemiluminescence, or silver staining. In addition, partial digestion and/or degradation of the genomic sample can be problematic. The disadvantage of pulsed field gel electrophoresis (PFGE) analysis is the need for sophisticated equipment and fresh cells or blood samples. Dosage analysis using polymorphic alleles depends on the informativeness of the marker. Although PCR-based microsatellite analysis is an improvement on previously used RFLP probes, the interpretation of results can be difficult, often requiring quantitative analysis, and is time-consuming in comparison with our method. FISH technology is complex and requires costly apparatus that is not always available in every DNA diagnostic laboratory. The development of rapid simple PCR-based tests for the diagnosis of CMT1A and HNPP would, therefore, help improve molecular diagnostic services such as prenatal or postnatal diagnostic tests.[23]

Haupt et al[20] described a PCR-based test using primers designed to exclusively amplify either the novel CMT1A or HNPP hybrid fragments. In their method, however, amplification products were also obtained from the nonrecombinant CMT1A-REP regions. Double restriction enzyme digestion of the PCR products was, therefore, required to distinguish between normal CMT1A and hybrid CMT1A-specific repeat (hybrid CMT1A-REP). These assays detected recombination events within the 1.7-kb hotspot located between an *Nsi*I site that is unique to the proximal CMT1A-REP and an *Eco*RI site that is unique to the distal CMT1A-REP. The expected detection rate within this region was 76.9% in CMT1A and 71.9% in HNPP patients.[26] Chang et al[27] described a similar test for CMT1A in which junction-specific primers were used to amplify the novel 3.2-kb hybrid fragments. No amplification product was produced in healthy individuals and in those patients with crossover breakpoints outside the 3.2-kb region. Although this produced reliable results, the workload per sample was still approximately twice that of the CMT1A test we describe here. Stronach et al[22] designed a PCR-based test that amplified a 3.6-kb region, including the 1.7-kb hotspot from specific CMT1A-REPs. Double restriction analysis was still required to distinguish normal and hybrid CMT1A-REPs. Although they extended the breakpoint region to 3.2 kb and increased the detection rate to 80% for CMT1A and 95% for HNPP,[22] they could not overcome the problem of nonspecific amplification.[18,22]

We used the allele-specific primers Hot-DF and Hot-PR for the CMT1A test, and Hot-PF and Hot-DR for the HNPP test, therefore ensuring specific amplification of the hybrid fragments only. This overcomes the problem of nonspecific amplification described by Haupt et al.[20]

It is important to note that the methods we used are expected to detect, at most, 82.5% of the patients with CMT1A duplication and 76% of the patients with HNPP deletion. A negative result, therefore, does not exclude the possibility of the diagnosis. In such cases, multiple unrelated techniques such as microsatellite,[17,24] Southern blot,[3,28] or MLPA[15] have to be employed in order to confirm or exclude duplication or deletion of a copy of the *PMP22* gene. The negative results may have resulted from infrequent variation in the CMT1A-REP sequences,[29–31] chimerism of the duplication junction fragments,[32] or possible recombination outside the CMT1A-REP sequences.[33,34] Additionally, as with any PCR-based technique used in a diagnostic setting, it is important to include suitable controls against PCR contamination, as well as positive and negative controls for the enzymatic digests.

In summary, in this study, stringently specific primers (Hot-PF, Hot-PR, Hot-DF, and Hot-DR) were used to overcome the problem of nonspecific amplification seen in other similar, previously published PCR-based techniques and provide a rapid, all-or-none and efficient screening test for CMT1A and HNPP.

Acknowledgment

We gratefully acknowledge a research grant from National Science Council, Taiwan, R.O.C. (NSC90-2314-B-075-058).

References

1. Skre H. Genetic and clinical aspects of Charcot-Marie-Tooth's disease. *Clin Genet* 1974;6:98–118.
2. Harding AE, Thomas PK. Genetic aspects of hereditary motor and sensory neuropathy (types I and II). *J Med Genet* 1980;17: 329–36.
3. Lupski JR, de Oca-Luna RM, Slaugenhaupt S, Pentao L, Guzzetta V, Trask BJ, Saucedo-Cardenas O, et al. DNA duplication associated with Charcot-Marie-Tooth disease type 1A. *Cell* 1991;66:219–32.
4. Raeymaekers P, Timmerman V, Nelis E, De Jonghe P, Hoogendijk JE, Baas F, Barker DF, et al. Duplication in chromosome 17p11.2 in Charcot-Marie-Tooth neuropathy type 1a (CMT 1a). The HMSN Collaborative Research Group. *Neuromuscul Disord* 1991;1:93–7.
5. Patel PI, Roa BB, Welcher AA, Schoener-Scott R, Trask BJ, Pentao L, Snipes GJ, et al. The gene for the peripheral myelin protein PMP-22 is a candidate for Charcot-Marie-Tooth disease type 1A. *Nat Genet* 1992;1:159–65.
6. Valentijn LJ, Bolhuis PA, Zorn I, Hoogendijk JE, van den Bosch N, Hensels GW, Stanton VP Jr, et al. The peripheral myelin gene PMP-22/GAS-3 is duplicated in Charcot-Marie-Tooth disease type 1A. *Nat Genet* 1992;1:166–70.
7. Timmerman V, Nelis E, Van Hul W, Nieuwenhuijsen BW, Chen KL, Wang S, Ben Othman K, et al. The peripheral myelin protein gene PMP-22 is contained within the Charcot-Marie-Tooth disease type 1A duplication. *Nat Genet* 1992;1:171–5.
8. Matsunami N, Smith B, Ballard L, Lensch MW, Robertson M, Albertsen H, Hanemann CO, et al. Peripheral myelin protein-22 gene maps in the duplication in chromosome 17p11.2 associated with Charcot-Marie-Tooth 1A. *Nat Genet* 1992;1: 176–9.
9. Nelis E, Van Broeckhoven C, De Jonghe P, Lofgren A, Vandenberghe A, Latour P, Le Guern E, et al. Estimation of the mutation frequencies in Charcot-Marie-Tooth disease type 1 and hereditary neuropathy with liability to pressure palsies: a European collaborative study. *Eur J Hum Genet* 1996;4: 25–33.
10. Hensels GW, Janssen EA , Hoogendijk JE, Valentijn LJ, Baas F, Bolhuis PA. Quantitative measurement of duplicated DNA as a diagnostic test for Charcot-Marie-Tooth disease type 1a. *Clin Chem* 1993;39:1845–9.
11. Cudrey C, Chevillard C, Le Paslier D, Vignal A, Passage E, Fontes M. Assignment of microsatellite sequences to the region duplicated in CMT1A (17p12): a useful tool for diagnosis. *J Med Genet* 1995;32:231–3.
12. Blair IP, Kennerson ML, Nicholson GA. Detection of Charcot-Marie-Tooth type 1A duplication by the polymerase chain reaction. *Clin Chem* 1995;41:1105–8.
13. Rautenstrauss B, Fuchs C, Liehr T, Grehl H, Murakami T, Lupski JR. Visualization of the CMT1A duplication and HNPP deletion by FISH on stretched chromosome fibers. *J Peripher Nerv Syst* 1997;2:319–22.
14. Ruiz-Ponte C, Loidi L, Vega A, Carracedo A, Barros F. Rapid real-time fluorescent PCR gene dosage test for the diagnosis of DNA duplications and deletions. *Clin Chem* 2000;46:1574–82.
15. Slater H, Bruno D, Ren H, La P, Burges T, Hills L, Nouri S, et al. Improved testing for CMT1A and HNPP using multiplex ligation-dependent probe amplification (MLPA) with rapid DNA preparations: comparison with the interphase FISH method. *Hum Mutat* 2004;24:164–71.
16. Pentao L, Wise CA, Chinault AC, Patel PI, Lupski JR. Charcot-Marie-Tooth type 1A duplication appears to arise from recombination at repeat sequences flanking the 1.5 Mb monomer unit. *Nat Genet* 1992;2:292–300.
17. Latour P, Boutrand L, Levy N, Bernard R, Boyer A, Claustrat F, Chazot G, et al. Polymorphic short tandem repeats for diagnosis of the Charcot-Marie-Tooth 1A duplication. *Clin Chem* 2001;47:829–37.
18. Reiter LT, Murakami T, Koeuth T, Pentao L, Muzny DM, Gibbs RA, Lupski JR. A recombination hotspot responsible for two inherited peripheral neuropathies is located near a mariner transposon-like element. *Nat Genet* 1996;12:288–97.
19. Lopes J, LeGuern E, Gouider R, Tardieu S, Abbas N, Birouk N, Gugenheim M, et al. Recombination hot spot in a 3.2-kb region of the Charcot-Marie-Tooth type 1A repeat sequences: new tools for molecular diagnosis of hereditary neuropathy with liability to pressure palsies and of Charcot-Marie-Tooth type 1A. French CMT Collaborative Research Group. *Am J Hum Genet* 1996;58:1223–30.
20. Haupt A, Schols L, Przuntek H, Epplen JT. Polymorphisms in the PMP-22 gene region (17p11.2-12) are crucial for simplified diagnosis of duplications/deletions. *Hum Genet* 1997;99: 688–91.
21. Yamamoto M, Keller MP, Yasuda T, Hayasaka K, Ohnishi A, Yoshikawa H, Yanagihara T, et al. Clustering of CMT1A duplication breakpoints in a 700 bp interval of the CMT1A-REP repeat. *Hum Mutat* 1998;11:109–13.
22. Stronach EA, Clark C, Bell C, Lofgren A, McKay NG, Timmerman V, Van Broeckhoven C, et al. Novel PCR-based diagnostic tools for Charcot-Marie-Tooth type 1A and hereditary neuropathy with liability to pressure palsies. *J Peripher Nerv Syst* 1999;4:117–22.
23. Bernard R, Labelle V, Negre P, Tardieu S, Azulay JP, Malzac P, Mattei JF, et al. 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. *Eur J Hum Genet* 2000;8:229–35.
24. LeGuern E, Ravise N, Gouider R, Gugenheim M, Lopes J, Bouche P, Agid Y, et al. 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* 1996;72:20–5.
25. Sambrook JFE, Maniatis T. *Molecular Cloning*, 2nd edition. New York: Cold Spring Harbor Laboratory Press, 1989.
26. Timmerman V, Rautenstrauss B, Reiter LT, Koeuth T, Lofgren A, Liehr T, Nelis E, et al. Detection of the CMT1A/HNPP recombination hotspot in unrelated patients of European descent. *J Med Genet* 1997;34:43–9.
27. Chang JG, Jong YJ, Wang WP, Wang JC, Hu CJ, Lo MC, Chang CP. Rapid detection of a recombinant hotspot associated with Charcot-Marie-Tooth disease type IA duplication by a PCR-based DNA test. *Clin Chem* 1998;44:270–4.
28. Lupski JR. DNA diagnostics for Charcot-Marie-Tooth disease and related inherited neuropathies. *Clin Chem* 1996;42: 995–8.
29. Reiter LT, Hastings PJ, Nelis E, De Jonghe P, Van Broeckhoven C, Lupski JR. Human meiotic recombination products revealed by sequencing a hotspot for homologous strand exchange in multiple HNPP deletion patients. *Am J Hum Genet* 1998;62: 1023–33.

30. Lopes J, Ravise N, Vandenberghe A, Palau F, Ionasescu V, Mayer M, Levy N, et al. 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* 1998;7:141–8.
31. Fuchs C, Liehr T, Ozbey S, Ekici A, Grehl H, Rautenstrauss B. Charcot-Marie-Tooth disease type 1A and hereditary neuropathy with liability to pressure palsies: a SacI polymorphism in the proximal CMT1A-REP elements may lead to genetic misdiagnosis. *Neurogenetics* 1998;2:43–6.
32. Lopes J, Tardieu S, Silander K, Blair I, Vandenberghe A, Palau F, Ruberg M, et al. Homologous DNA exchanges in humans can be explained by the yeast double-strand break repair model: a study of 17p11.2 rearrangements associated with CMT1A and HNPP. *Hum Mol Genet* 1999;8:2285–92.
33. Valentijn LJ, Baas F, Zorn I, Hensels GW, de Visser M, Bolhuis PA. Alternatively sized duplication in Charcot-Marie-Tooth disease type 1A. *Hum Mol Genet* 1993;2:2143–6.
34. Vandenberghe A, Latour P, Chauplannaz G, Chapon F, Pouget J, Dumas R, Laguenay A, et al. Molecular diagnosis of Charcot-Marie-Tooth 1A disease and hereditary neuropathy with liability to pressure palsies by quantifying CMT1A-REP sequences: consequences of recombinations at variant sites on chromosomes 17p11.2-12. *Clin Chem* 1996;42:1021–5.