

Effectiveness of Real-Time Quantitative PCR Compare to Repeat PCR for the Diagnosis of Charcot-Marie-Tooth Type 1A and Hereditary Neuropathy with Liability to Pressure Palsies

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The majority of cases of Charcot-Marie-Tooth type 1A (CMT1A) and of hereditary neuropathy with a liability to pressure palsies (HNPP) are the result of heterozygosity for the duplication or deletion of peripheral myelin protein 22 gene (PMP22) on 17p11.2. Southern blots, pulsed-field gel electrophoresis (PFGE), fluorescence in situ hybridization (FISH) and polymorphic marker analysis are currently used diagnostic methods. But they are time-consuming, labor-intensive and have some significant limitations. We describe a rapid real-time quantitative PCR method for determining gene copy number for the identification of DNA duplication or deletion occurring in CMT1A or HNPP and compare the results obtained with REP-PCR. Six patients with CMT1A and 14 patients with HNPP [confirmed by Repeat (REP)-PCR], and 16 patients with suspicious CMT1A and 13 patients with suspicious HNPP [negative REP-PCR], and 15 normal controls were studied. We performed REP-PCR, which amplified a 3.6 Kb region (including a 1.7 Kb recombination hotspot), using specific CMT1A-REP and real-time quantitative PCR on the LightCycler system. Using a comparative threshold cycle (Ct) method and β -globin as a reference gene, the gene copy number of the PMP22 gene was quantified. The PMP22 duplication ratio ranged from 1.35 to 1.74, and the PMP22 deletion ratio from 0.41 to 0.53. The PMP22 ratio in normal controls ranged from 0.81 to 1.12. All 6 patients with CMT1A and 14 patients with HNPP confirmed by REP-PCR were positive by real-time quantitative PCR. Among the 16 suspicious CMT1A and 13 suspicious HNPP with negative

REP-PCR, 2 and 4 samples, respectively, were positive by real-time quantitative PCR. Real-time quantitative PCR is a more sensitive and more accurate method than REP-PCR for the detection of PMP22 duplications or deletions, and it is also faster and easier than currently available methods. Therefore, we believe that the real-time quantitative method is useful for diagnosing CMT1A and HNPP.

Key Words: Charcot-Marie-Tooth type 1A, HNPP, peripheral myelin protein 22 gene, real-time quantitative PCR

INTRODUCTION

The majority of Charcot-Marie-Tooth 1(CMT1) patients possess DNA rearrangements, which are the molecular causes of their disease. A 1.5-Mb tandem duplication, the CMT1A duplication, accounts for approximately 70 percent of CMT1 cases, and a deletion of the same 1.5-Mb region in chromosome 17p11.2 is found in >85 percent of patients with HNPP. The CMT1A duplication and HNPP deletion result from unequal crossing-over and reciprocal homologous recombination involving a 24-Kb repeat-CMT1A-REP-which flanks the 1.5-Mb region. A meiotic recombination hotspot occurs within CMT1A-REP. The majority of the *de novo* duplication and deletion events occur during male germ cell meiosis. The CMT1A and HNPP phenotypes result from a gene dosage effect. CMT1A is due to trisomic overexpression of the peripheral myelin protein-22 gene (PMP22) whereas HNPP results from the monosomic un-

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derexpression of PMP22. Moreover, in rare cases PMP22 point mutations can cause disease in patients without the CMT1A duplication or the HNPP deletion.¹

In molecular genetic testing for the CMT1A duplication and the HNPP deletion, several approaches are available. Molecular diagnosis by hybridization-based methods include; Southern blotting,²⁻⁵ quantitative photostimulated luminescence (PSL) imaging,⁶⁻⁹ and pulse-field gel electrophoresis (PFGE).^{2,5,10-12} Less frequently used methods include fluorescence-in situ hybridization (FISH),^{13,14} polymorphic markers,^{12,15} repeat (REP-) PCR,¹⁶⁻¹⁸ and endpoint quantitative PCR.^{19,20} But, these methods have several disadvantages for the diagnosis of CMT1A and HNPP.

In this study, we describe a rapid real-time quantitative PCR devised to assess gene copy number for the identification of DNA duplication or deletion occurring in CMT1A or HNPP, and we compare the results obtained with REP-PCR.

MATERIALS AND METHODS

Subjects and DNA extraction

Six patients with CMT1A and 14 patients with HNPP (confirmed by REP-PCR), 16 suspicious CMT1A and 13 suspicious HNPP patients with negative REP-PCR, and 15 normal controls were studied. Genomic DNA was extracted from EDTA-treated blood using the MagNa Pure LC DNA isolation kit and instrument (Roche), according to the manufacturer's instructions. DNA analysis was carried out with 'informed consent'.

REP-PCR

REP-PCR was performed as described by Stronach et al.¹⁷ The method amplifies a 3.6 Kb region, including the recombination 1.7 Kb hot-spot, from specific CMT1A-REP. A schematic of the method is shown in Fig. 1.

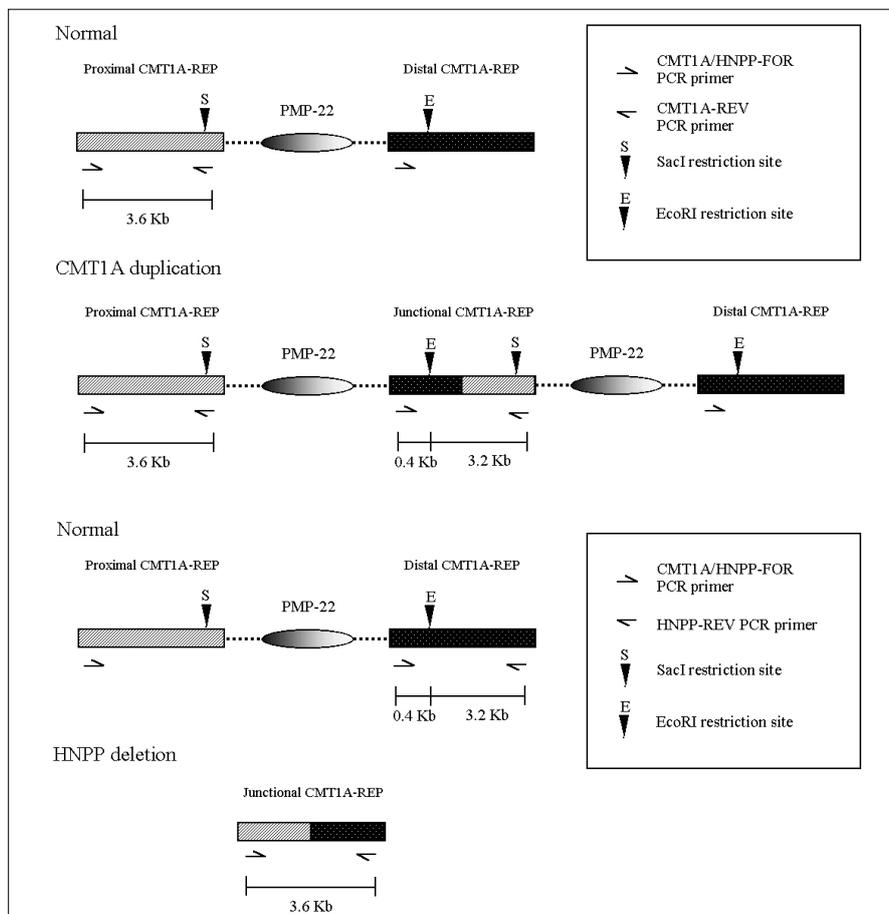


Fig. 1. Schematic method of REP-PCR. The junctional CMT1A-REPs shown exhibit crossing-over within the 3.2 Kb region.

Real-time quantitative PCR

Primers and probes used for real-time quantitative PCR were designed using LightCycler Probe Design Software version 1.0 (Roche). The amplified fragment (202 bp), including exon 5 of the PMP22 gene, was obtained using forward primer 5'-ACC CAG TGC ATC CAA C-3' and reverse primer 5'-ATC CAT AGC ACC ATT TCA AAG-3'. The sequence of the sensor probe was 5'-CGG CTT GTG GCA TTG GCA-Fluorescein-3', and the sequence of the anchor probe 5'-LCRed 640-CTT GCC CTT ACA GGT GGA GTA TCT-3'. The primers and probes of the β -globin gene in LightCycler Control DNA kit (Roche) were used as reference. Amplification was carried out using the LightCycler FastStart DNA Master Hybridization Probe kit (Roche) in a standard PCR containing; 0.5 μ mol/L of each primer and 0.2 μ mol/L of each probe in a 20 μ L final volume containing 2 μ L of sample. The cycling program consisted of an initial denaturation at 95°C for 10 min, and 45 cycles of 95°C for 10 sec, 58°C for 10 sec, and 72°C for 9 sec. Amplifications of PMP22 and β -globin were performed simultaneously in each capillary tube. Each test was repeated three times.

Calculating the gene copy number

Using a comparative threshold cycle (Ct) method, as previously described by Livak,²¹ and β -globin as a reference gene, the gene copy number by PMP22 gene duplication or deletion was quantified and calculated.

RESULTS

The amplification curves for PMP22 and the β -globin gene are shown in Fig. 2. The Ct values of PMP22 and of the β -globin gene were almost the same in the normal sample, whereas the Ct values of PMP22 in CMT1A and HNPP were decreased or increased, respectively, versus that of β -globin in the amplification curve. The PMP22 duplication ratio in two CMT1A patients, confirmed by REP-PCR, ranged from 1.35 to 1.74, the PMP22 deletion ratio in 14 patients with HNPP, as confirmed by REP-PCR, ranged from 0.41 to 0.53 and the PMP22

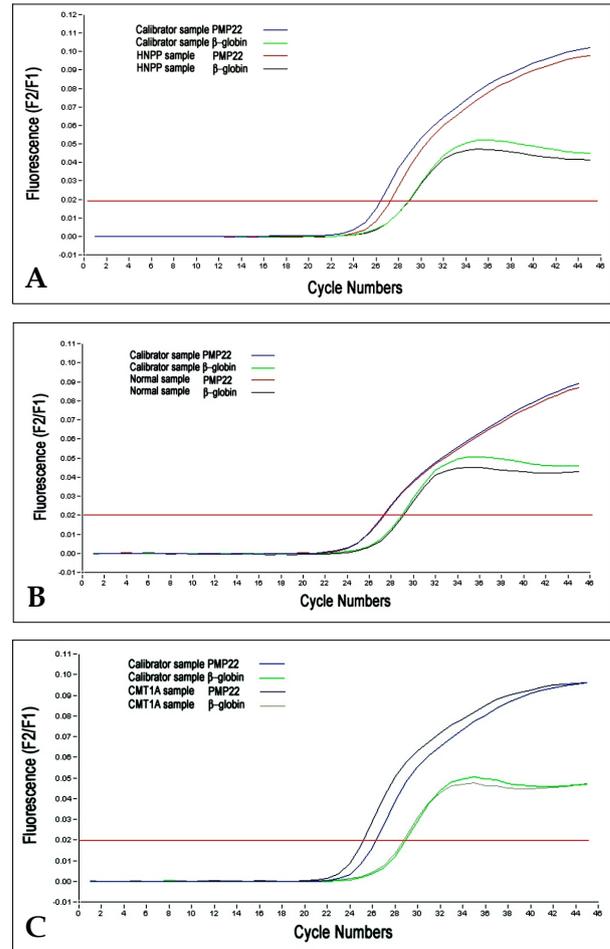


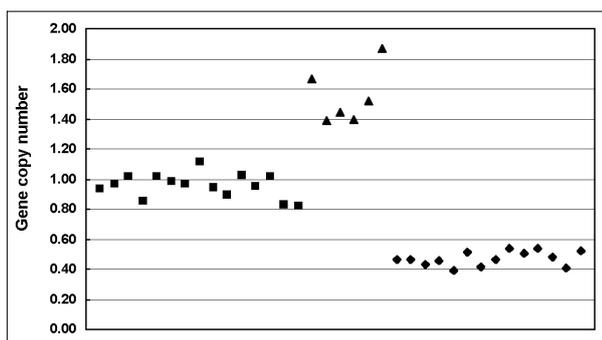
Fig. 2. Amplification curves of PMP22 and of the β -globin gene in HNPP (A), normal (B) and CMT1A (C) subjects. In normal sample, the Ct values of PMP22 and of the β -globin gene were almost the same (middle), whereas the Ct values of PMP22 in HNPP and CMT1A were increased (upper) or decreased (lower), respectively, versus that of β -globin in the amplification curve.

ratio in the controls ranged from 0.81 to 1.12 (Table 1). The standard deviations of Ct were 0.22, 0.18 and 0.24 in CMT1A, HNPP and controls, respectively. Six patients with CMT1A and the 14 patients with HNPP, as confirmed by REP-PCR, were positive by real-time quantitative PCR. Among the 16 suspicious CMT1A and 13 suspicious HNPP patients, with a negative REP-PCR, 2 and 4 samples, respectively, were positive by real-time quantitative PCR. Therefore, the real-time quantitative PCR method can be more detected 50% (2/4) of the CMT1A patients and 22.2% (4/18) of the HNPP patients with cross-over in CMT1A-REP. The measured copy numbers of the

Table 1. Peripheral Myelin Protein 22 (PMP22) Duplication and Deletion Ratios Detected by Real Time Quantitative PCR in CMT1A and HNPP and in Normal Controls, Confirmed by REP PCR

Samples	PMP22 ratio		
	Mean	Range	SD* of Ct value
Control (n= 15)	0.95	0.81-1.12	0.24
CMT1A (n=6)	1.55	1.35-1.74	0.22
HNPP (n=14)	0.47	0.41-0.53	0.18

*Standard deviation.

**Fig. 3.** Measured copy numbers of the PMP22 gene per haploid genome in 15 normal controls (■), 6 CMT1A (▲) and 14 HNPP (◆). No overlap of gene copy number between each groups.

15 normal controls, and in the 2 CMT1A and 14 HNPP patients are shown in Fig. 3. No overlap of copy number was found between CMT1A or HNPP patients and the controls.

DISCUSSION

At least nine loci for CMT1 and four for CMT2 have been identified by genetic linkage studies. Six of the genes associated with CMT1, and recently one gene for CMT2, has been identified. Although at least eight loci are associated with the aCMT1 phenotype, 70 to 90% of patients possess the causative CMT1A duplication. Deletion of the same genomic segment that is duplicated in CMT1A leads to a clinically distinct neuropathy-HNPP.¹ Recombination events occur within the 1.7 Kb hotspot located between an *EcoRI* site, unique to the proximal CMT1A-REP, and a *NsiI* site, unique to distal CMT1A-REP. It has been reported that the recombination rate in hotspot region ranges from 75 to 87% in a population-

dependent manner.¹ In the present study, the recombination rate in the hotspot region was about 73% (16/22), which is similar to that obtained in other populations.

Several methods for detecting the CMT1A duplication and the HNPP deletion are available currently. Recently, the real-time quantitative PCR method has been used to detect haploidy and triploidy in genetic diseases and cancer.²²⁻²⁵ Detection of the PMP22 duplication and deletion by real-time quantitative PCR using TaqMan probes has been known as a fast method allowing to be diagnosed in 2 hr. However, specific probes are required in real-time quantitative PCR using TaqMan probes.

In the present study, this method also can be more found 27% (6/22) of duplication and deletion and is more sensitive than REP-PCR. However, many more samples should be tested for CMT1A duplication ratio. For mutation studies in the PMP22 gene, P0 gene (CMT1B), connexin32 gene (CMT1X),²⁶ or another subtype of CMT is needed in samples without duplication or deletion.²⁷ One case of CMT1A patient with 1.87 of gene copy number was presumed homozygote duplication, but it should be confirmed by other reference method e.g. FISH.

The reaction efficiencies varied in the different experiments, thus it may not be possible to obtain similar efficiencies for both control and target markers. This limitation also affects highly accurate systems such as the LightCycler instrument. But the gene dosage test in the method described is feasible and more accurate. In this study, the mean CV of the Ct value of the PMP22 and β -globin genes was < 2.0% in between run. Therefore, it appears that results of real-time quantitative PCR were accurate and reproducible.

In conclusion, real-time quantitative PCR is both more sensitive and more accurate than REP-PCR in terms of the detection of PMP22 duplications or deletions. It is also faster and easier than other currently available methods and clearly differentiates gene dose. Therefore, real-time quantitative PCR method may be useful for diagnosing CMT1A and HNPP, and may be applied to the diagnosis of any disease caused by gene dosage changes.

REFERENCES

- Lupski JR, Garcia CA. Charcot-Marie-Tooth peripheral neuropathies and related disorders. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. *The metabolic & molecular bases of inherited disease*. 8th ed. New York: McGraw-Hill; 2001. p.5759-88.
- Lupski JR, de Oca-Luna RM, Slaugenhaupt S, Pentao L, Guzzetta V, Trask BJ, et al. DNA duplication associated with Charcot-Marie-Tooth disease type 1A. *Cell* 1991; 66:219-32.
- MacMillan JC, Upadhyaya M, Harper PS. Charcot-Marie-Tooth disease type 1a (CMT1a): evidence for trisomy of the region p11.2 of chromosome 17 in south Wales families. *J Med Genet* 1992;29:12-3.
- Raeymaekers P, Timmerman V, Nelis E, de Jonghe P, Hoogendijk JE, Baas F, et al. The HMSN Collaborative Research Group: Duplication in chromosome 17p11.2 in Charcot-Marie-Tooth neuropathy type 1a (CMT 1a). *Neuromuscul Disord* 1991;1:93-7.
- Raeymaekers P, Timmerman V, Nelis E, van Hul W, de Jonghe P, Martin JJ, et al. Estimation of the size of the chromosome 17p11.2 duplication in Charcot-Marie-Tooth neuropathy type 1a (CMT1a). HMSN Collaborative Research Group. *J Med Genet* 1992;29:5-11.
- Chen KL, Wang YL, Dodson LA, Rennert H, Mochan BS, Wilson R, et al. Normalized Southern hybridization to enhance testing for Charcot-Marie-Tooth disease, type 1A. *Mol Diagn* 1996;1:65-71.
- Hoogendijk JE, Hensels GW, Gabreels-Festen AA, Gabreels FJ, Janssen EA, de Jonghe P, et al. De novo mutation in hereditary motor and sensory neuropathy type I. *Lancet* 1992;339:1081-2.
- Hoogendijk JE, Janssen EA, Gabreels-Festen AA, Hensels GW, Joosten EM, Gabreels FJ, et al. Allelic heterogeneity in hereditary motor and sensory neuropathy type 1a (Charcot-Marie-Tooth disease type 1A). *Neurology* 1993;43:1010-5.
- Ikegami T, Ikeda H, Chance PF, Kiyosawa H, Yamamoto M, Sobue G, et al. Facilitated diagnosis of CMT1A duplication in chromosome 17p11.2-12: analysis with a CMT1A-REP repeat probe and photostimulated luminescence imaging. *Hum Mutat* 1997;9:563-6.
- Chance PF, Abbas N, Lensch MW, Pentao L, Roa BB, Patel PI, et al. Two autosomal dominant neuropathies result from reciprocal DNA duplication/deletion of a region on chromosome 17. *Hum Mol Genet* 1994;3:223-8.
- 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.
- Timmerman V, Lofgren A, le Guern E, Liang P, de Jonghe P, Martin JJ, et al. Molecular genetic analysis of the 17p11.2 region in patients with hereditary neuropathy with liability to pressure palsies (HNPP). *Hum Genet* 1996;97:26-34.
- Roa BB, Greenberg F, Gunaratne P, Sauer CM, Lubinsky MS, Kozma C, et al. Duplication of the PMP 22 gene in 17p partial trisomy patients with Charcot-Marie-Tooth type-1 neuropathy. *Hum Genet* 1996;97: 642-9.
- Shaffer LG, Kennedy GM, Spikes AS, Lupski JR. Diagnosis of CMT1A duplications and HNPP deletions by interphase FISH: implications for testing in the cytogenetics laboratory. *Am J Med Genet* 1997;69:325-31.
- Navon R, Timmerman V, Lofgren A, Liang P, Nelis E, Zeitune M, et al. Prenatal diagnosis of Charcot-Marie-Tooth disease type 1A (CMT1A) using molecular genetic techniques. *Prenat Diagn* 1995;15:633-40.
- 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.
- Stronach EA, Clark C, Bell C, Lofgren A, McKay NG, Timmerman V, 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.
- Yamamoto M, Keller MP, Yasuda T, Hayasaka K, Ohnishi A, Yoshikawa H, et al. Clustering of CMT1A duplication breakpoints in a 700-bp interval of the CMT1A-REP repeat. *Hum Mutat* 1998;11:109-13.
- Poropat RA, Nicholson GA. Determination of gene dosage at the PMP22 and androgen receptor loci by quantitative PCR (comments). *Clin Chem* 1998;44:724-30.
- Young P, Stogbauer F, Wiebusch H, Lofgren A, Timmerman V, van Broeckhoven C, et al. PCR-based strategy for the diagnosis of hereditary neuropathy with liability to pressure palsies and Charcot-Marie-Tooth disease type 1A. *Neurology* 1998;50:760-3.
- Livak KJ. Comparative Ct method. ABI Prism 7700 Sequence Detection System. User Bulletin No. 2, PE Applied Bio-systems; 1997
- Thiel CT, Kraus C, Rauch A, Ekici AB, Rautenstrauss B, Reis A. A new quantitative PCR multiplex assay for rapid analysis of chromosome 17p11.2-12 duplications and deletions leading to HMSN/HNPP. *Eur J Hum Genet* 2003;11:170-8.
- Lorentzos P, Kaiser T, Kennerson ML, Nicholson GA.

- A rapid and definitive test for Charcot-Marie-Tooth 1A and hereditary neuropathy with liability to pressure palsies using multiplexed real-time PCR. *Genet Test* 2003;7:135-8.
24. Kim SW, Lee KS, Jin HS, Lee TM, Koo SK, Lee YJ, et al. Rapid Detection of duplication/deletion of the PMP 22 gene in patients with Charcot-Marie-Tooth disease type 1A and hereditary neuropathy with liability to pressure palsy by real-time quantitative PCR using SYBR Green I Dye. *J Korean Med Sci* 2003;18:727-32.
 25. Kim YR, Choi JR, Song KS, Chong WH, Lee HD. Evaluation of HER2/neu status by real-time quantitative PCR in breast cancer. *Yonsei Med J* 2002;43:335-40.
 26. Nelis E, Haites N, Van Broeckhoven C. Mutations in the peripheral myelin genes and associated genes in inherited peripheral neuropathies. *Hum Mutat* 1999;13: 11-28.
 27. De Visser M, Van Broeckhoven C, Nelis E. Hereditary motor and sensory neuropathy or Charcot-Marie-Tooth disease type 1A and 1B, 2nd ed. Royal Soc Med, London/Eur Neuromuscular Centre. London: Royal Society of Medicine Press; 1997. p.49-52.