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Rapid Detection of Duplication/Deletion of the *PMP22* 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

Mutations and altered gene dosage of the peripheral myelin protein (*PMP22*) gene in chromosome 17p11.2-12 are the main causes for hereditary neuropathies, accounting for approximately 70% of all cases. Patients with duplication of the *PMP22* develop Charcot-Marie-Tooth disease type 1A (CMT1A) and deletion of one *PMP22* allele leads to hereditary neuropathy with liability to pressure palsy (HNPP). Twenty patients with CMT1A, 17 patients with HNPP, and 18 normal family members and 28 normal controls were studied by real-time quantitative PCR using SYBR Green I on the ABI 7700 Sequence Detection System. The copy number of the *PMP22* gene was determined by the comparative threshold cycle method and the *albumin* was used as a reference gene. The *PMP22* duplication ratio ranged from 1.45 to 2.06 and the *PMP22* deletion ratio ranged from 0.42 to 0.64. The *PMP22* ratio in normal controls, including normal family members, ranged from 0.85 to 1.26. No overlap was found between patients with CMT1A or patients with HNPP and normal controls. This method is fast, highly sensitive, specific, and reproducible in detecting *PMP22* duplication and deletion in CMT1A and HNPP patients, respectively.

Key Words : *Charcot-Marie-Tooth disease; Hereditary neuropathy with liability to pressure palsy; Reactions, polymerase chain SYBR Green*

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INTRODUCTION

Charcot-Marie-Tooth (CMT) disease is a clinically and genetically heterogeneous group of hereditary peripheral neuropathies. The clinical characteristics of the disease include distal symmetric muscle weakness, atrophy, bilateral pes cavus, and diminished or absent deep tendon reflexes. Peripheral nerve conduction velocities are often severely reduced. In hereditary neuropathy with liability to pressure palsy (HNPP), recurrent peripheral nerve palsies (e.g., ulnar nerve, median nerve, and peroneal nerve palsies) occur because of minor compression trauma, whereas foot deformity is less frequently observed than it is in CMT. Nerve conduction velocities are significantly reduced at compression sites of peripheral nerves.

Charcot-Marie-Tooth disease type 1A (CMT1A) is the most common form of CMT (1, 2). About 70% of CMT1A cases are caused by a dominantly inherited 1.5-Mb duplication at 17p11.2-12, encompassing the peripheral myelin protein-22 gene (*PMP22*) (3-5). In contrast, deletion of the *PMP22* characteristically results in HNPP. Point mutation in the *PMP22* may result in CMT1A or HNPP. Thus heterozygous carriers of the deletion (HNPP) or duplication (CMT1A) have one or three copies of the *PMP22*, respectively. For molecular diagnosis of duplication or deletion of the *PMP22*, several approaches including Southern blot analysis (6), fluorescence in situ hybridization (FISH) (7, 8), pulsed-field gel electrophoresis (PFGE) (4, 9), polymorphic short tandem repeats (STR) analysis (7, 10, 11), and endpoint quantitative PCR (12, 13) are available.

STR-PCR methods have been widely used because of its advantages in cost, labor, amount of DNA sample required, and turnaround time. However, there is a limitation in sensitivity (14), and DNA samples of parents are required in marker typing. Detection of the *PMP22* duplication and deletion by real-time quantitative PCR using TaqMan probes has been reported (15-17). The method is fast, allowing 13 patients to be diagnosed in 2 hr. However, specific probes are required in real-time quantitative PCR using TaqMan probes, but not in real-time quantitative PCR using SYBR Green I. Real-time quantitative PCR using SYBR Green I has been used previously for quantification of specific human genes (18, 19), as well as detection and quantification of DNA of virus, bacteria, and fungus (20-23). However, there was no study on detecting the *PMP22* duplication and deletion by real-time

quantitative PCR using SYBR Green I dye. In this study, we used real-time quantitative PCR using SYBR Green I to detect the *PMP22* duplication and deletion. Using a comparative Ct (threshold cycle) method, the relative gene copy number was quantified (16, 17).

MATERIALS AND METHODS

Subjects

The study subjects were 20 patients with CMT1A, 17 patients with HNPP, 18 asymptomatic normal family members, and 28 normal unrelated controls. Clinical and neurophysiological examinations were performed on all patients. Duplication or deletion of the *PMP22* gene in CMT1A or HNPP patients was diagnosed previously by short tandem repeats (STR) analysis with three polymorphic markers, *D17S122, D17S162,* and *D17S261*. Informed consents were obtained from all individuals participating in this study.

DNA extraction

Genomic DNA was extracted from leukocytes of EDTA-treated blood using the QIAamp DNA blood kit (QIAGEN). The concentration and purity of the preparations were determined by measuring the absorbance at 260 and 280 nm. DNAs were diluted in distilled water to a concentration of 1.25 ng/μL and stored at -20℃ until use.

Primer design and real-time quantitative PCR

The primer sequences were as follows; PMP22 exon 3 forward primer, 5′-TCTGTCCAGGCCACCATGA-3′; PMP22 exon 3 reverse primer, 5′-GAAGAGTTGGCAGAAGAACAGGA-3′; human serum albumin exon 12 forward primer, 5′-TGTTGCATGAGAAAACGCCA-3′; human serum albumin exon 12 reverse primer, 5′ -GTCGCCTGTTCACCAAGGAT-3′. A 3-by-3-primer matrix (combinations of 1 μM, 5 μM, and 10 μM of each forward and reverse primers) was analyzed to determine the optimal concentrations of both forward and reverse primers. The minimum primer concentrations that resulted in the lowest Ct-value (threshold cycle) and highest fluorescent signal (△Rn), while minimizing non-specific amplification, were chosen as an optimal pair. The amplifications were carried out in a 96-well plate in a 20 μL reaction volume containing 10 μL SYBR Green PCR Master Mix (Applied Biosystems, Foster City, Califonia, U.S.A.), 5 μM (1 μL) forward primer, 5 μM (1 μL) reverse primer, and 10 ng (8 μL) of genomic DNA. In each assay, 2 or more normal controls and no-template control were included. Each sample was run in triplicate for both *PMP22* and *albumin*. The 96-well sample tray was centrifuged briefly at 3,000 rpm for 2 min. PCR reactions were run in the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Preincubation was performed for 10 min at 95℃ to denature the target DNA and activate AmpliTaq Gold DNA Polymerase. DNA was amplified for 40 cycles of 15 sec at 95℃ and 1 min at 65℃. The annealing and extension temperatures were optimized for specificity of the PCR product. The fluorescence signal

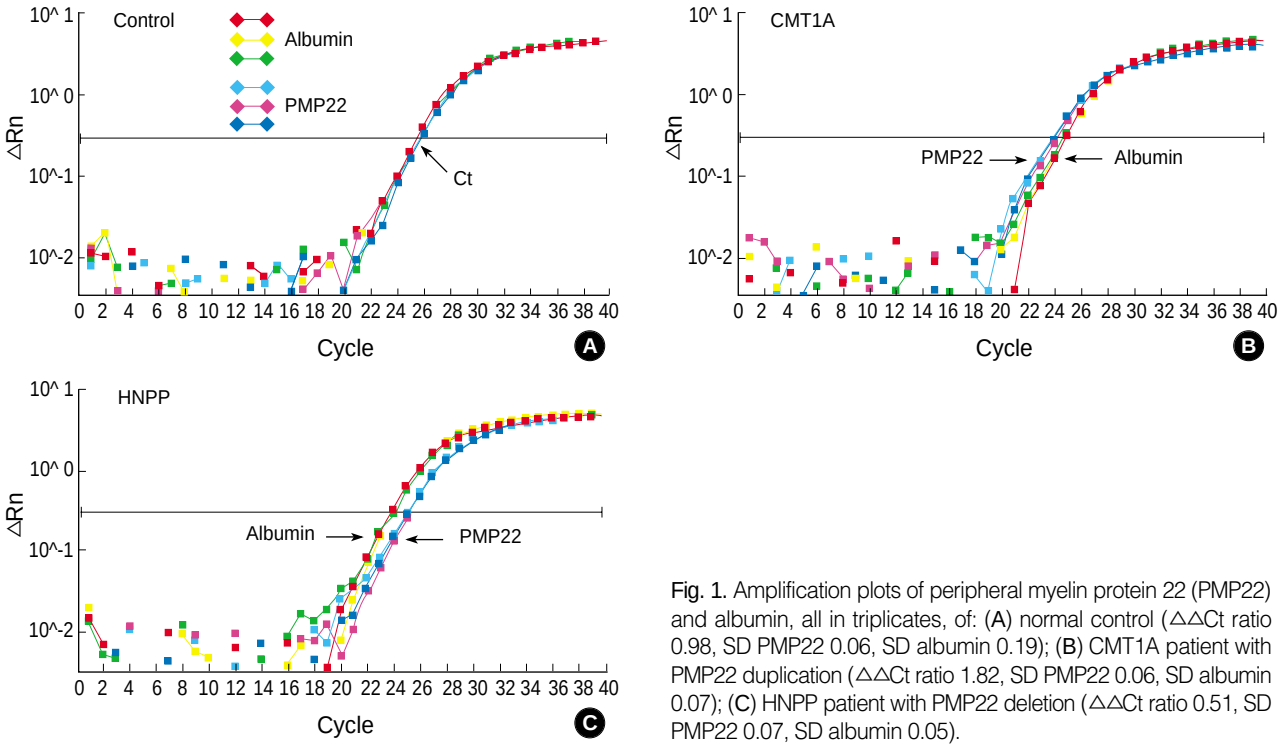


Fig. 1. Amplification plots of peripheral myelin protein 22 (PMP22) and albumin, all in triplicates, of: (A) normal control (△△Ct ratio 0.98, SD PMP22 0.06, SD albumin 0.19); (B) CMT1A patient with PMP22 duplication (△△Ct ratio 1.82, SD PMP22 0.06, SD albumin 0.07); (C) HNPP patient with PMP22 deletion (△△Ct ratio 0.51, SD PMP22 0.07, SD albumin 0.05).

was measured at the end of the elongation phase. We used MicroAmp Optical Caps and MicroAmp Optical 96-Well Reaction Plates (Applied Biosystems).

Data analysis

The data was analyzed by using the ABI PRISM Sequence Detection System and Microsoft Excel. The relative gene copy number was quantified by the comparative Ct method (16, 17). Amplicons were run as triplicates in separate tubes to permit quantification of the *PMP22* gene normalized to *albumin*, an endogenous gene, as a control. By using a calibrator sample of normal control DNA, the gene copy number of unknown samples was estimated. The patients, families, and normal controls were analyzed in parallel with the calibrator sample on each assay plate. The threshold cycle number (Ct) was determined for all PCR reactions. The Ct parameter represents the cycle number (△Rn) at which the amplification plot, representing the fluorescence emission of the reporter dye, passed a fixed threshold. The threshold was automatically set at 10 standard deviations (SD) above the mean baseline emission. However, the threshold was manually adjusted within the logarithmic curve, above the background level (calculated from cycles 3-15) and below the plateau phase. The same threshold and baseline were set for all samples including the calibrator samples. Using the comparative Ct method, the starting copy number of the samples was determined: △Ct represents the mean Ct value of each sample and was calculated for *PMP22* and *albumin*. The starting copy number of the unknown samples was determined relative to the known copy number of the calibrator sample using the following formula: DDCt=[△Ct albumin (calibrator sample)-△Ct PMP22 (calibrator sample)]-[△Ct albumin (unknown sample)-△Ct PMP22 (unknown sample)]. The relative gene copy number was calculated by the expression $2^{-\triangle\triangle Ct}$. This formula was based on the assumption that the rate of Ct change versus the rate of target DNA copy change was identical for both the *PMP22*

Table 1. *PMP22* gene copy number (relative ratio) detected by real-time quantitative PCR using SYBR Green I dye

Samples	△△Ct ratio		
	Mean	Range	SD
Normal (*n*=46)	1.09	0.85-1.26	0.09
CMT1A (*n*=20)	1.66	1.45-2.06	0.16
HNPP (*n*=17)	0.53	0.42-0.64	0.06

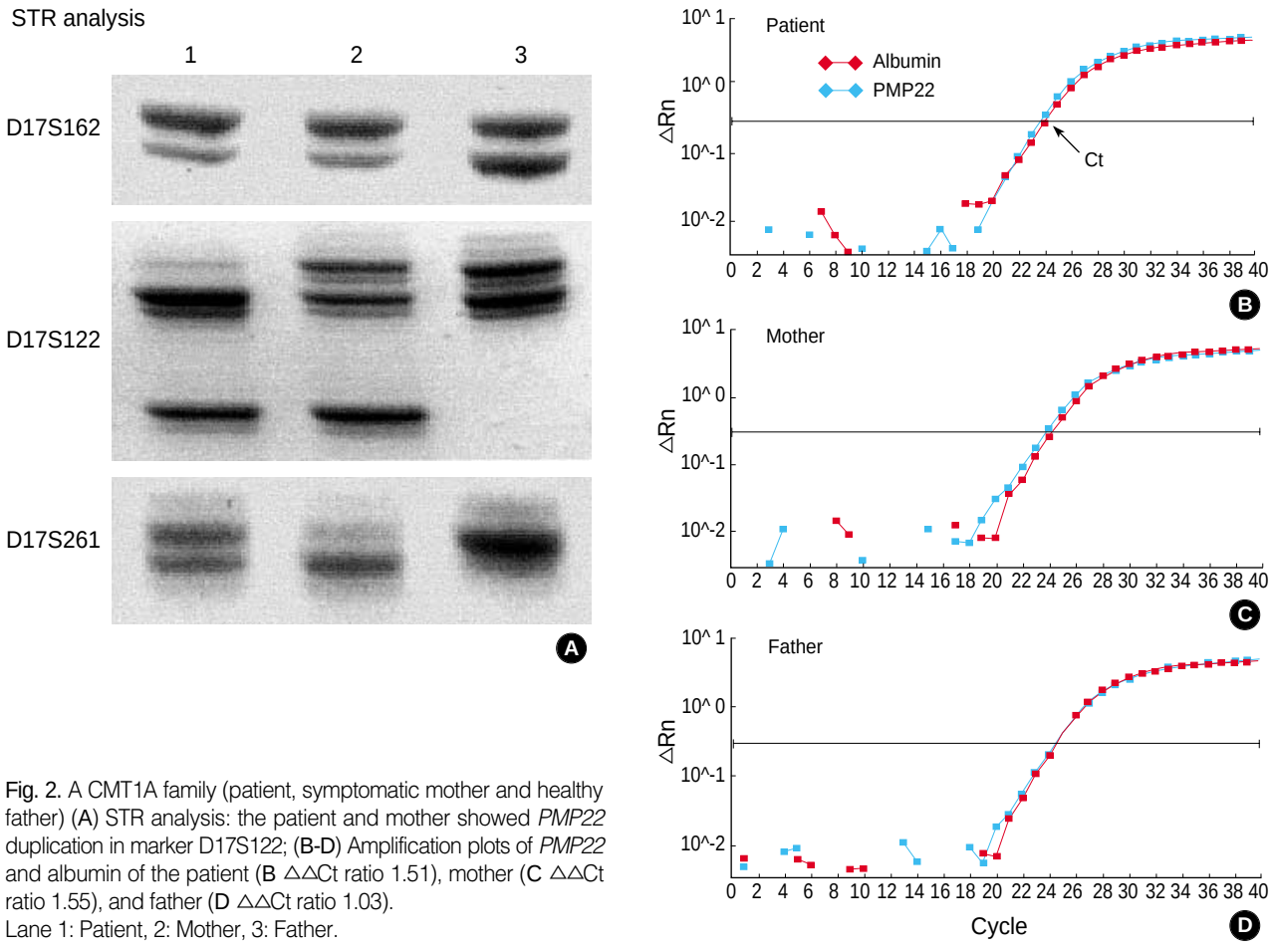
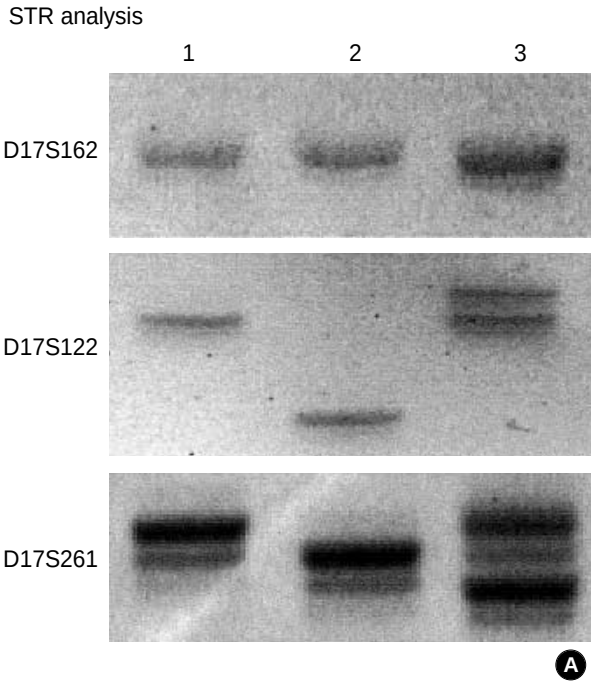


Fig. 2. A CMT1A family (patient, symptomatic mother and healthy father) (A) STR analysis: the patient and mother showed *PMP22* duplication in marker D17S122; (B-D) Amplification plots of *PMP22* and albumin of the patient (B △△Ct ratio 1.51), mother (C △△Ct ratio 1.55), and father (D △△Ct ratio 1.03).
Lane 1: Patient, 2: Mother, 3: Father.



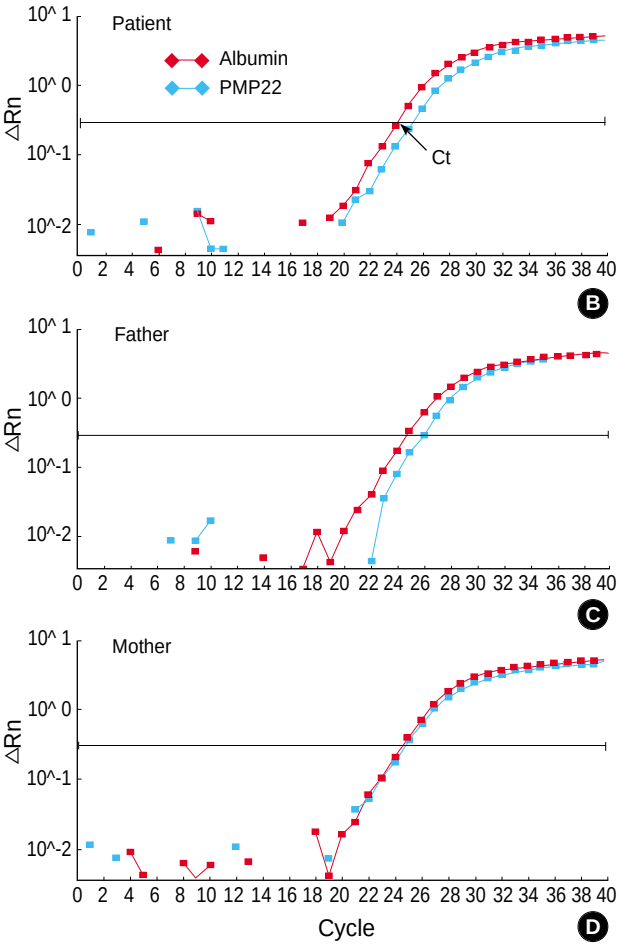


Fig. 3. A HNPP family (patient, symptomatic father and healthy mother) (A) STR analysis: the patient showed *PMP22* deletion in marker D17S122 and D17S261; (B-D) Amplification plots of *PMP22* and albumin of the patient (B △△Ct ratio 0.51), father (C △△Ct ratio 0.50), and mother (D △△Ct ratio 1.06).
Lane 1: Patient, 2: Mother, 3: Father.

gene of interest and *albumin*. Using this method, a △△Ct ratio is expected to be about 1 in normal controls, about 0.5 in patients with HNPP and about 1.5 in patients with CMT1A.

RESULTS

The triplicate runs of *PMP22* and *albumin* showed almost complete overlap of parallel amplification plots. For all samples the SD of Ct values was very low (mean, 0.09; range, 0.01-0.33). The PCR amplification plots of a normal control sample (Fig. 1A) showed a slightly increased but nearly identical Ct value of *PMP22* triplicates, compared with that of *albumin*. In samples positive for the *PMP22* duplication (CMT1A), the Ct value of *PMP22* showed a decrease of about 0.5, compared with that of *albumin* (Fig. 1B), whereas, in samples positive for the *PMP22* deletion (HNPP), the Ct value of *PMP22* showed an increase of about 1.2, compared with that of *albumin* (Fig. 1C).

Table 1 shows the △△Ct ratios [$2^{-(\Delta\Delta Ct)}$] in three groups. The mean △△Ct ratio in normal controls, including asymptomatic normal family members, was 1.09 (range, 0.85-1.26). The mean △△Ct ratio in CMT1A patients was 1.66 (range, 1.45-2.06). The mean △△Ct ratio in HNPP patients was 0.53 (range, 0.42-0.64). Real-time quantitative PCR analysis results of one CMT1A and HNPP case confirmed by STR analysis showed correct determination of the PMP22 gene copy number (Fig. 2 and 3).

DISCUSSION

For detection of duplication and deletion of *PMP22* gene, Southern blot analysis or STR marker typing have been widely used. However, these methods have several limitations. Southern blot analysis is not only time-consuming work but also requires large amount of patient's DNA. Although for STR marker typing, small DNA samples are sufficient for the procedure but DNA samples of parents are necessary for interpretation.

The recently developed real-time quantitative PCR is fast and precise, and avoids the use of hazardous radioisotopes (15-17, 24). In real-time quantitative PCR assay, the initial copy number of the target template is determined by analyzing the cycle-to-cycle change in fluorescence signal as a result of the amplification of the template during PCR. The fewer cycles

it takes to reach a detectable level of fluorescence, the greater the initial copy number. Detection of the *PMP22* duplication and deletion by real-time quantitative PCR (15-17) are fast and sensitive. The method takes only 2 hr and does not require post-PCR processing. In addition, DNA samples of parents are not required. Real-time quantitative PCR using SYBR Green I dye will be more convenient and cost effective than using expensive TaqMan probes. We now established real-time quantitative PCR using SYBR Green I dye for fast, cheap, and reliable detection of the gene copy number of the *PMP22* gene.

We studied 20 CMT1A and 17 HNPP patients by real-time quantitative PCR using SYBR Green I dye, which confirmed previously for duplication or deletion of the PMP22 gene by STR analysis. The PCR amplification plots of the *PMP22* gene of normal control samples showed slightly increased but nearly identical Ct values compared with *albumin*. By the comparative Ct method, the relative *PMP22* gene copy number was determined. The lower and upper limits of *PMP22* duplication ratio in CMT1A patients were 1.45 and 2.06, respectively. This result indicated that the *PMP22* gene copy number in CMT1A patients was three or four. The lower and upper limits of *PMP22* deletion ratio in HNPP patients were 0.42 and 0.64, respectively. This result indicated that the *PMP22* gene copy number in HNPP patients was one. In 28 normal controls and 18 normal family members, the *PMP22* ratio ranged from 0.85 to 1.26. No overlap was observed between CMT1A or HNPP patients and normal controls.

In conclusion, real-time quantitative PCR using SYBR Green I is highly sensitive, specific, and reproducible in detecting *PMP22* duplication and deletion in patients with CMT1A and HNPP. This method will be very helpful for the diagnosis of patients with CMT1A/HNPP as well as genetic counseling for their family.

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