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ORIGINAL ARTICLE

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A rapid and reliable detection system for the analysis of *PMP22* gene dosage by MP/DHPLC assay

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Abstract Charcot-Marie-Tooth disease type 1A (CMT1A) and hereditary neuropathy with liability to pressure palsies (HNPP) are caused by a 1.5-Mb duplication and a deletion at chromosome 17p11.2–12 encompassing the peripheral myelin protein 22 gene (*PMP22*), respectively. We developed a rapid and reliable detection system for duplications/deletions of the *PMP22* gene based on measurement of gene copy number. The method involves amplification of a test locus with unknown copy number and a reference locus of known copy number by multiplex PCR (MP), followed by denaturing high-performance liquid chromatography (DHPLC) or capillary electrophoresis detection to identify single copy changes. Thirty-two patients with CMT1A, 17 patients with HNPP, and 61 unaffected individuals were analyzed. Using the same competitive MP protocol, the measured *PMP22* gene dosage revealed concordant results between DHPLC and capillary electrophoresis analysis. The results of the MP/DHPLC or the MP/capillary electrophoresis assay were all confirmed by PCR–restriction fragment length polymorphism analysis. We concluded that the MP/DHPLC assay is an efficient, accurate, and reliable technique for gene dosage determination of the *PMP22* gene for CMT1A duplication and HNPP deletion. This technique further extends the application of DHPLC as an alternative method for the measurement of gene amplifications and heterozygous deletions in different genetic diseases.

Keywords Denaturing high-performance liquid chromatography · Capillary electrophoresis · CMT1A · HNPP · *PMP22* gene · Gene dosage

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Introduction

Although most diseases are caused by point mutations, in many cases, genomic duplications or deletions of genes have also been shown to be responsible for the same disorder. Changes in gene copy number may lead to under- or over-expression of those genes, resulting in a disease phenotype (Lynch 2002). Determination of gene copy number has thus become more important in molecular genetic diagnosis.

Charcot-Marie-Tooth (CMT) disease is the most common inherited disorder of the peripheral nervous system (1/2,500) (Skre 1974). Skre (1974) gives a phenotypic description and a discussion of the genetic heterogeneity of CMT disease type 1, where CMT1A is the most common form of CMT. Around 70% of CMT1A cases are associated with a 1.5-Mb duplication at chromosome 17p11.2–12, where the peripheral myelin protein 22 gene (*PMP22*) is located (Lupski et al. 1991; Patel et al. 1992; Pentao et al. 1992; Raeymaekers et al. 1991; Timmerman et al. 1992). In contrast, hereditary neuropathy with liability to pressure palsies (HNPP) is caused by a reciprocal deletion of the same region

(Chance et al. 1993). In a minority of cases, point mutations in *PMP22* can also result in CMT1A and HNPP (Boerkoel et al. 2002).

Many methods to determinate the gene copy number of the *PMP22* gene have been reported. Molecular testing by hybridization-based methods includes Southern blotting (Ikegami et al. 1997; MacMillan et al. 1992), pulsed field gel electrophoresis (Lupski et al. 1991; Pentao et al. 1992; Timmerman et al. 1996), and fluorescence in situ hybridization (Lupski et al. 1991; Roa et al. 1996). Other approaches, including analysis of restriction fragment length polymorphism (RFLP) and microsatellite inheritance, have also been commonly used (Badano et al. 2001; Latour et al. 2001; Navon et al. 1995; Roa et al. 1996; Timmerman et al. 1996).

Hybridization-based techniques have several common problems, including the need to use labeled probes and large amounts of DNA together with time-consuming methods. Although microsatellite analysis is a powerful PCR-based technique, its application is limited to cases where parental DNA is available for analysis and where polymorphic markers within the deleted region turn out to be informative in the individual case. Another frequently used method is real-time PCR using standard PCR in conjugation with the fluorescence TaqMan method (Laurendeau et al. 1999; Wilke et al. 2000). However, there are also disadvantages to this approach. Since the results from real-time PCR provide no means of resolving size, the number of loci that can be analyzed in a single tube is currently restricted to four or less by fluorescence profiles and the optical properties of the hardware. Therefore, in applications where multiple loci must be examined, results can only be achieved in multiple tubes, and may be inaccurate due to pipetting errors.

In this study, we introduce a newly developed multiplex PCR (MP)/denaturing high-performance liquid chromatography (DHPLC) assay to determine the gene copy number of *PMP22* gene in an efficient way. The assay uses the X-linked *CYBB* gene and autosomal *KRIT1* gene as the standards to determine the relative gene dosage of the *PMP22* gene. We demonstrate that this assay is able to accurately distinguish two gene copies from both one copy and three copies; it can thus identify CMT1A patients, HNPP patients, and normal individuals by the accurate determination of *PMP22* gene copy number using the DHPLC system.

Materials and methods

Sample preparation

All study subjects were individuals with clinical manifestations of CMT1A/HNPP based on clinical examination and electrophysical diagnosis as carried out previously at National Taiwan University Hospital (Pan et al. 2003; Wilke et al. 2000). Genomic DNA was extracted from peripheral whole blood using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions.

RFLP analysis

All unrelated CMT1A, HNPP patients, and normal control individuals were confirmed by a method previously described (Stronach et al. 1999). The method was a PCR-based test that amplifies a 3.6-kb region including the 1.7-kb hotspot from specific CMT type 1A-repetitions (CMT1A-REPs), allowing the rapid diagnosis of CMT1A and HNPP patients. In the analysis of 32 CMT1A and 17 HNPP patients, duplication and deletion events were detected in all samples with cross-over breakpoints known to be within the region amplified by PCR. Primers were designed using a published sequence proximal to the distal CMT1A-REP sequence (GenBank accession numbers: U41165-distal and U41166-proximal). Primers for the CMT1A test were, CMT1A/HNPP forward (5′-AGGTTGTTTACTCCTTCTTC-3′), and CMT1A reverse (5′-AGATGGAATAGTAGAGCTCAC-3′). Primers for the HNPP test were, CMT1A/HNPP forward (sequence as above), and HNPP reverse (5′-AGATGGAATAGTAGAGTGAG-3′). The amplifications for the CMT1A and HNPP tests were carried out in 50 μl volumes with 1.5 mM $MgCl_2$, 0.4 μM of the appropriate primers, 80 μM dNTPs, 100 ng genomic DNA, 2.6 U *Taq* DNA polymerase using the Expand Long Template PCR system (Roche Applied Science, Mannheim, Germany). Amplification for CMT1A and HNPP testing comprised an initial denaturation step at 94°C for 3 min followed by ten cycles of 30 s at 94°C, 30 s at 54°C, 3 min at 68°C; 25 cycles of 30 s at 94°C, 30 s at 54°C, 3 min at 72°C, including a 20 s auto-extend function on the extension time to give a final extension of 7 min at 68°C using a multiblock system (MBS) thermocycler (ThermoHybaid, Ashford, UK). Amplified products were digested with *Eco*RI (New England Biolabs, Beverly, MA) at 37°C for 1 h and electrophoresed at 80 V on 0.8% agarose gels.

Multiplex PCR amplification

Primers for MP amplification were designed to amplify three target sequences, one or two of which lie within the potentially duplicated or deleted target region, the others being reference sequences. Primers were designed to have a similar T_m (the temperature at which 50% of double-stranded DNA is denatured) values and sequence composition to allow the use of similar conditions for the MP system. In the case of quantitation of X chromosome copy number, the *CYBB* gene was coamplified in the same tube in which quantitation of the X chromosome copy number for males and females was carried out. The *KRIT1* gene was another genomic reference and was also coamplified in the same tube to confirm the result.

To amplify the *PMP22*, *CYBB*, and *KRIT1* genes, primer sequences were as follows: *PMP22* exon 3

forward (5′-CTTCTGCTTCTGCTGCCTGT-3′) and reverse (5′-CATTCTGAGGCCACATCCTT-3′), *PMP22* exon 4 forward (5′-CTAGGTGGCCAAGATTGGAA-3′) and reverse (5′-GGGATTTTGGGCTAGCTCTT-3′), *CYBB* exon 13 forward (5′-CGGGAAATTCACC-TACTTGC-3′) and reverse (5′-AGCATTATTTGAG-CATTTGGC-3′), and *KRIT1* exon 8 forward (5′-TTC GAATGGCTACTTCTACCTG-3′) and reverse (5′-AA AACGTCTTTTAAATCAGAGC-3′). The X-linked *CYBB* gene and *KRIT1* gene were used as controls to determine the relative gene dosage of the *PMP22* gene. The final volume of the MP was 25 μl containing 100 ng genomic DNA, Set 1 used 0.08 μM of each primer of *CYBB* and *KRIT1* genes, 0.2 μM of the primer of exon 3 of *PMP22* gene; Set 2 used 0.08 μM of each primer of *CYBB* and *KRIT1* genes, 0.2 μM of the primer of exon 4 of *PMP22* gene; Set 3 used 0.08 μM of the primer of *KRIT1* gene, 0.28 μM of the primer of exon 3 and 0.24 μM of the primer of exon 4 of *PMP22* gene; 200 μM dNTPs, 0.5 U AmpliTaq Gold enzyme (PE Applied Biosystems, Foster City, CA), and 2.5 μl GeneAmp 10× buffer II (10 mM Tris–HCl, pH 8.3, 50 mM KCl), in 2 mM $MgCl_2$ as provided by the manufacturer. Amplification was performed in an MBS thermocycler (ThermoHybaid). PCR amplification was carried out with an initial denaturation step at 95°C for 10 min, followed by 25 cycles consisting of the denaturation at 94°C for 30 s, the annealing at 56°C for 45 s, the extension at 72°C for 45 s, and a final extension step at 72°C for 10 min.

For capillary electrophoresis analysis, the MP protocol described above with the same primer pairs and condition was applied except that fluorescently labeled primer pairs were used and the cycle number was reduced to 23 cycles.

Capillary electrophoresis and DHPLC analysis

For capillary electrophoresis analysis, 3 μl fluorescently labeled product was mixed well with a mixture containing the Rox-labeled G-500 marker and HiDi Formamide (1:40). This mixture was denatured for 5 min at 95°C. Using a four-color-laser-induced capillary electrophoresis system (ABI Prism 310 Genetic Analyzer), polymer GeneScan POP 4, 1× Genetic Analyzer buffer with EDTA (PE Applied Biosystems), a 47-cm uncoated capillary (Chromatographie Service, Langerwehe, Germany), and sample injection at 15 kV for 5 s, separation of the PCR products was reliably achieved at 15 kV over 26 min.

The DHPLC system used to analyze non-fluorescently labeled PCR products was a Transgenomic Wave Nucleic Acid Fragment Analysis System (Transgenomic, San Jose, CA). DHPLC was carried out on automated HPLC instrumentation equipped with a DNASep column (Transgenomic). The DNASep column contained proprietary 2-mm non-porous alkylated poly(styrenedivinylbenzene) particles. The DNA molecules that elute from the column are detected by scanning with a UV detector at 260 nm. DHPLC grade acetonitrile (9017–03, JT Baker, Phillipsburg, NJ) and triethylammonium acetate (TEAA; Transgenomic, Crewe, UK) were used to constitute the mobile phase. The mobile phases consisted of 0.1M TEAA with 500 μl acetonitrile (eluent A) and 25% acetonitrile in 0.1M TEAA (eluent B). The start- and end-points of the gradient were adjusted according to the size of the PCR products by an algorithm provided by the WAVEmaker system control software (Transgenomic). PCR product (15 μl) was injected for each analysis run. The samples were separated at 50°C. The buffer B gradient increased by 2%/min for 4.5 min at a flow rate of 0.9 ml/min. Generally, the analysis for each injection took about 10 min.

Finally, DHPLC and GeneScan software were used to record peak heights corresponding to the signal from each PCR product.

Data analysis

All MP amplifications were carried out in triplicate for each sample tested. Two target reference PCR product ratios were calculated from each triplicate MP. For known unaffected control samples, the mean of the two ratio values was calculated, and each was scaled to a value of 1.0. All test sample ratios were then scaled accordingly.

To calculate the dosage quotient of the *PMP22* gene copy number from unknown samples (U) in comparison with control samples (C), the following formulae were used:

$$\frac{\text{Peak Height of } PMP22 \text{ Gene (U)}/[\text{Peak Height of } CYBB \text{ Gene (U)} \times \text{K (U)}]}{\text{Peak Height of } PMP22 \text{ Gene (C)}/[\text{Peak Height of } CYBB \text{ Gene (C)} \times \text{K (C)}]} \times 2$$

$$\frac{\text{Peak Height of } PMP22 \text{ Gene (U)}/[\text{Peak Height of } KRIT1 \text{ Gene (U)} \times \text{K (U)}]}{\text{Peak Height of } PMP22 \text{ Gene (C)}/[\text{Peak Height of } KRIT1 \text{ Gene (C)} \times \text{K (C)}]} \times 2$$

Because the *CYBB* gene is an X-linked gene, we introduce *K*(U) and *K*(C) to represent factors for the unknown and control samples: male = 2 and female = 1.

Results

In this study, 17 patients with HNPP, 32 patients with CMT1A, and 61 normal controls were analyzed. All diagnoses for duplication or deletion of the *PMP22* gene were confirmed by PCR–RFLP analysis (Stronach et al. 1999). Based on the method for capillary electrophoresis analysis of Yau et al. (1996), we could determine the gene dosage of the *PMP22* gene from genomic references comprising one gene on an autosomal chromosome (*KRIT1* gene, on chromosome 7) and one on the X chromosome (*CYBB* gene, X-linked). By capillary electrophoresis analysis, we could unambiguously identify all samples with different *PMP22* gene copies (Fig. 1).

To further explore the possible application of DHPLC to gene dosage analysis of the *PMP22* gene, we used the same competitive MP protocol that we had applied previously to the capillary electrophoresis system. In order to determine the optimal PCR cycling number for MP/DHPLC analysis, we ran a series of tests with incremental increases in cycle number (21, 23, 25, 27 cycles). Chromatography using DHPLC of samples with incremental PCR cycle numbers is shown in Fig. 2. All of the results show that the reactions were in linear phase, and 25 cycles was chosen as the number of cycles for quantitative analysis.

Chromatography using DHPLC analysis of the various combinations of competitive MP is shown in Fig. 3. The MP/DHPLC protocol is similar to that of capillary electrophoresis analysis, and the values for peak height of the autosomal *KRIT1* gene and the X-linked *CYBB* gene were used as genomic references to determine the *PMP22* gene copy number. We demonstrated that the dosage of *PMP22* gene could be identified correctly by DHPLC analysis, using either the autosomal *KRIT1* gene or the X-linked *CYBB* gene as a genomic reference (Fig. 4). Using both DHPLC and capillary electrophoresis, the measured *PMP22* gene copy number was between 1.5 and 2.5 for the normal population, below 1.5 for HNPP patients and above 2.5 for CMT1A patients. In this study, there was no overlapping or ambiguous data observed for either DHPLC or capillary electrophoresis.

Table 1 lists the expected and calculated copy numbers of the *PMP22* gene as determined using both capillary electrophoresis and DHPLC analysis for the samples, including all the patients with HNPP, CMT1A, and the normal controls. The correlation of the calculated *PMP22* gene copy number for all the samples tested between the capillary electrophoresis and DHPLC analysis is shown in Fig. 5, and the correlation coefficient is 0.923 ($P < 0.01$).

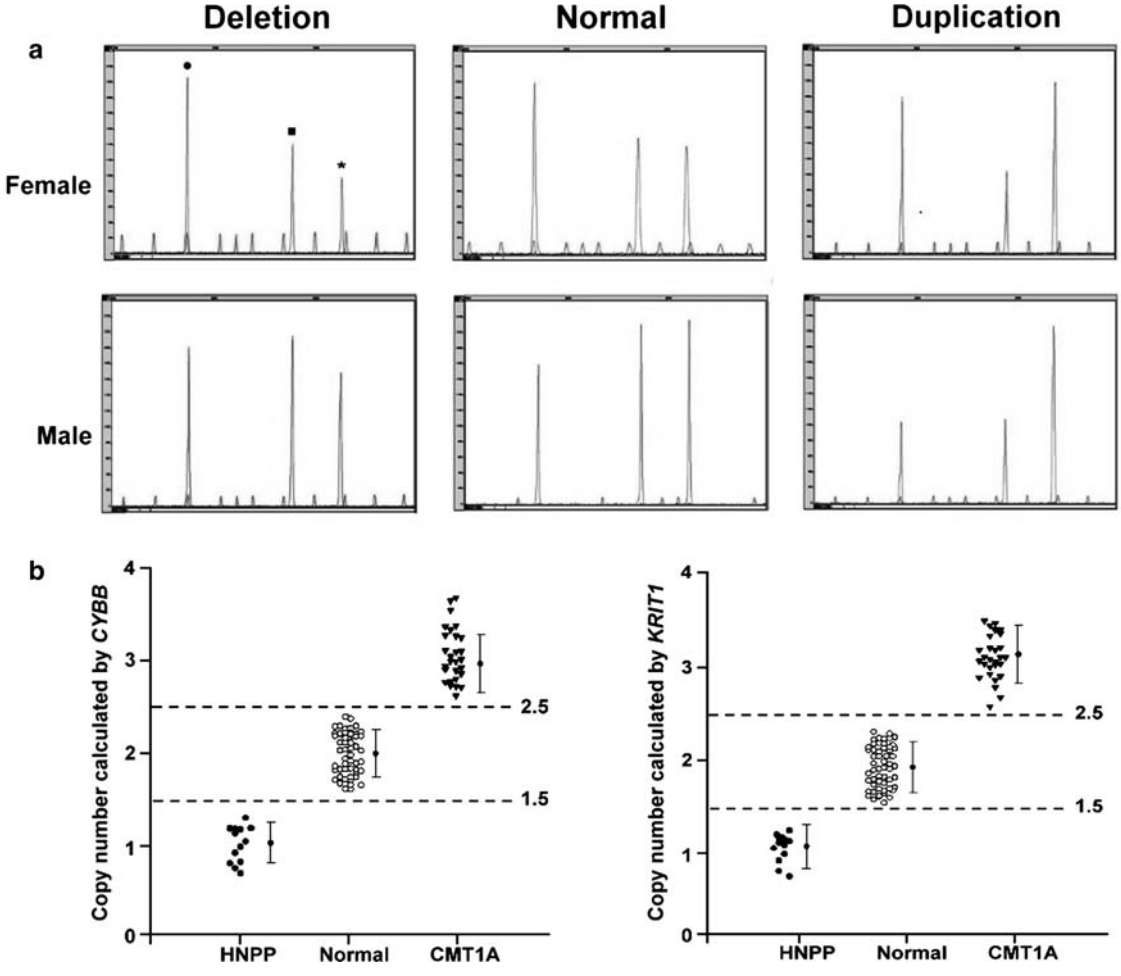

Fig. 1 Use of capillary electrophoresis analysis to identify *PMP22* gene copy number. **a** Electropherogram of the multiplex PCR (MP) in Set 1 (*KRIT1*/*CYBB*/exon 3 of *PMP22* gene) of DNA samples from the Charcot-Marie-Tooth disease type 1A (CMT1A) (duplication), hereditary neuropathy with liability to pressure palsies (HNPP) (deletion), and normal control. **b** Scatter plot of capillary electrophoresis results for CMT1A, HNPP, and normal controls. ● *CYBB* gene (X-linked), ■ *KRIT1* gene, ★ exon 3 of *PMP22* gene, ⌶ mean ± SD

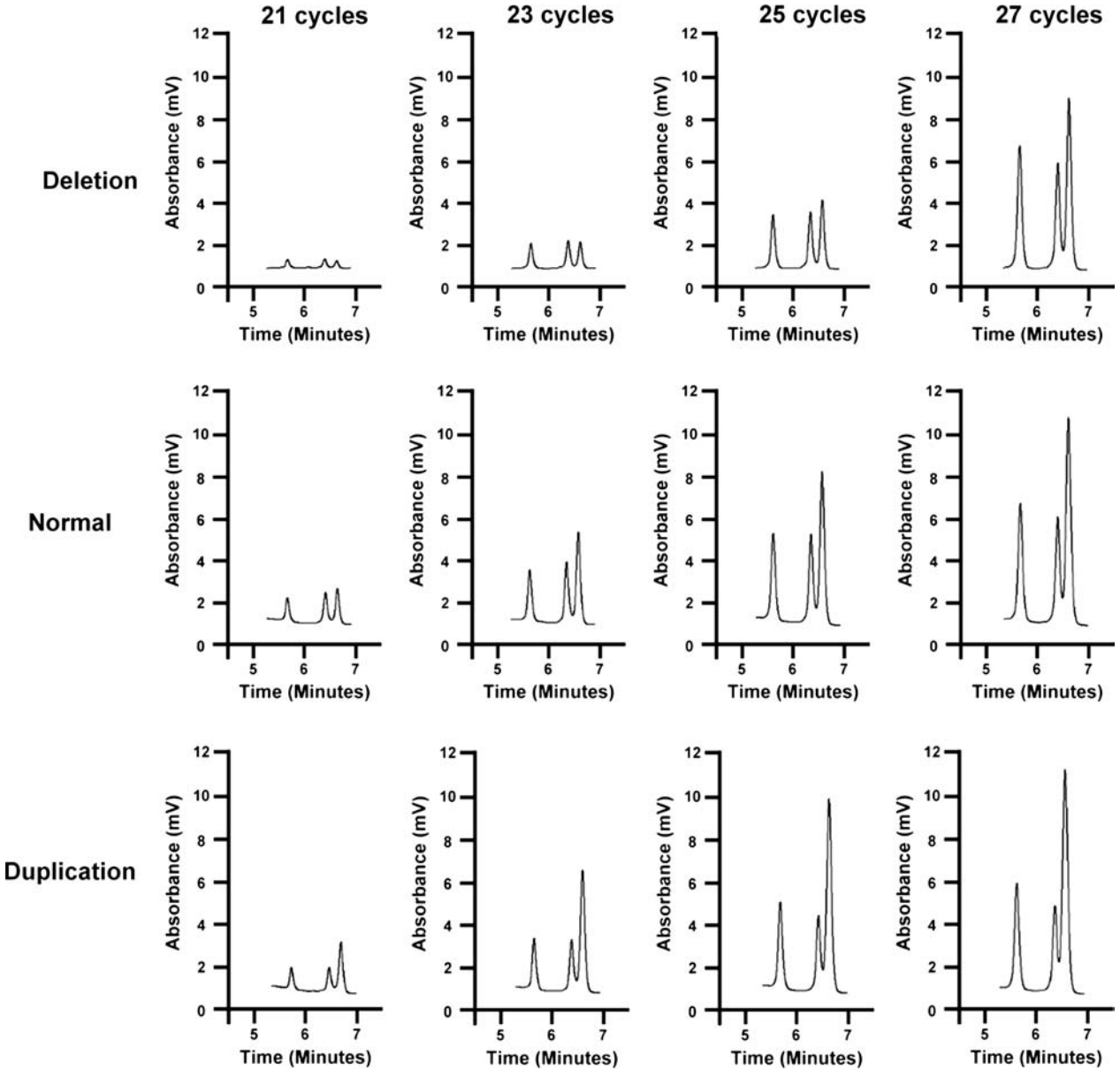

Fig. 2 Determining PCR cycle number. Using incremental increases in the PCR cycle number from 21 to 27 cycles, the gene ratios for DNA samples from the CMT1A patients (duplication), HNPP patients (deletion), and normal controls are constant

Comparing the time needed for these three methods, PCR–RFLP analysis required 5 h for the long-range PCR and 30 min for enzyme treatment, while capillary electrophoresis required 2 h for the PCR and about 90 min to analyze the data. However, DHPLC was the quickest, requiring 2 h for the PCR and only 30 min for data analysis.

Discussion

Since CMT1A and HNPP are frequent autosomal dominant disorders of inherited peripheral neuropathy, it is important to identify *PMP22* gene dosage for diagnostic purpose and genetic counseling. Various methods have been described to quantify gene/exon copy numbers, including microarray hybridization (Zhu et al. 2003), real-time PCR (Kim et al. 2003; Thiel et al. 2003), hybridization with amplifiable probes, and auto-electrophoresis analysis (Akrami et al. 2003; Frisso et al. 2004; Rowland et al. 2001; Yau et al. 1996). Although these methods are reliable, they also carry some limitations when applied to clinical situations. Most of the major problems are time, expense, radio-isotope/fluorescence probe labeling, and the need for large amounts of DNA. Hence, we have developed a

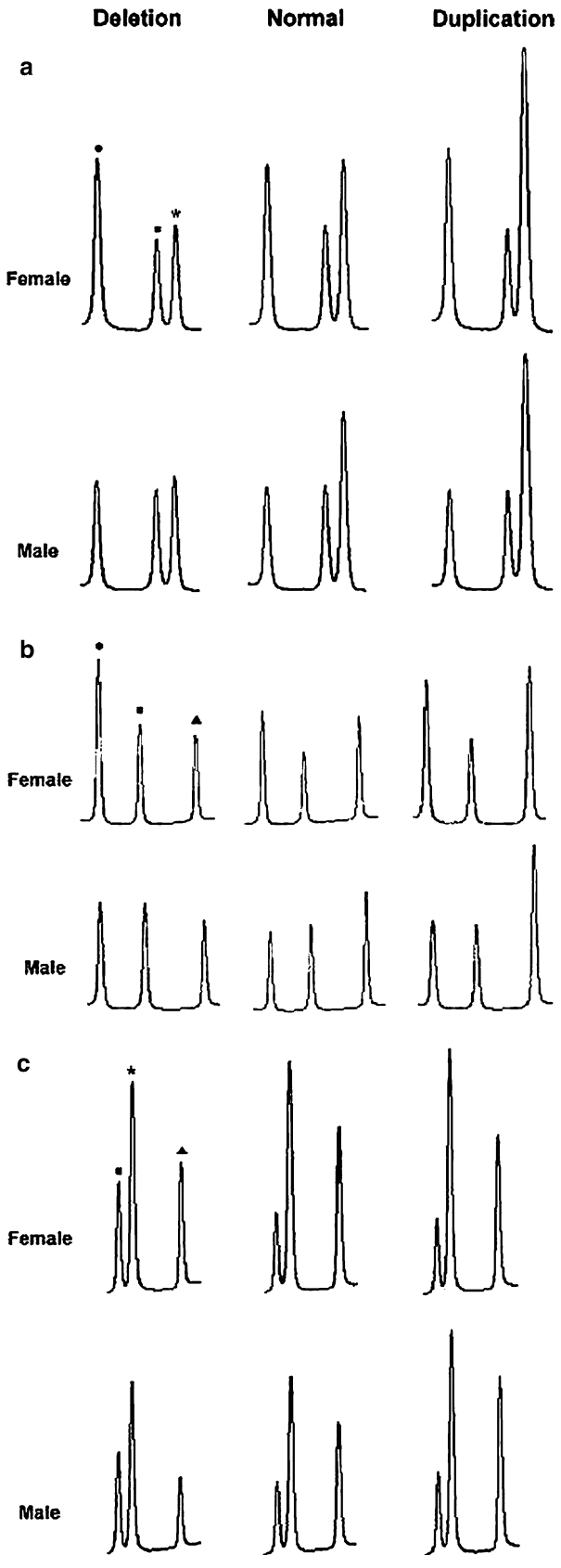

Fig. 3a–c Chromatography of denaturing high-performance liquid chromatography (DHPLC) analysis of three sets of DNA samples from two unaffected controls (one male and one female) compared with DNA from two patients with CMT1A (duplication) or with HNPP (deletion). **a** MP Set 1 (*CYBB*/*KRIT1*/exon 3 of *PMP22* gene). **b** MP Set 2 (*CYBB*/*KRIT1*/exon 4 of *PMP22* gene), **c** MP Set 3 (*KRIT1*/exon 3 of *PMP22* gene/exon 4 of *PMP22* gene). ● *CYBB* gene (X-linked), ■ *KRIT1* gene, ★ exon 3 of *PMP22* gene, ▲ exon 4 of *PMP22* gene

rapid multiplex competitive PCR-based quantitative technique for relative DNA quantitation (Su et al. 2005).

In this study, the MP approach is a relatively quantitative method. We utilize one or two genomic references to determine the copy number of the gene to be examined: quantitation of the target gene is relative to reference genes of known copy number (Celi et al. 1993; Dehainault et al. 2004; Noonan et al. 1990). A fragment from within a potentially duplicated or deleted target region is amplified simultaneously with a disomic reference region in an MP system. Based on the observation that the amount of PCR product generated from each site of amplification is proportional to the amount of starting template, the ratio of the amount of PCR product generated from each amplification reaction indicates whether there is duplication, deletion, or no change in the target area. Detection of PCR products is carried out either by DHPLC or capillary electrophoresis, both of which provide the sensitivity required for detection of single-copy dosage changes.

A major advantage of this MP approach is that the analysis is carried out within a single tube; therefore, any factors influencing the PCR will affect both reactions in a similar manner and will not alter the final ratio. An additional advantage of the technique is the coupling of DHPLC/capillary electrophoresis and detection in a single system, which is suitable for large-scale sample analysis.

To calculate *PMP22* gene dosage by competitive MP strategy, we designed primer pairs for the *PMP22* gene to amplify exon 3 and exon 4 in combination, using two other genes (one autosomal *KRIT1* gene, one X-linked *CYBB* gene) as the genomic references. Three independent primer sets with different combinations between these four PCR fragments (*KRIT1*/*CYBB*/exon 3 of *PMP22* gene; *KRIT1*/*CYBB*/exon 4 of *PMP22* gene; *KRIT1*/exon 3 of *PMP22* gene/exon 4 of *PMP22* gene) were evaluated to test the validity of the assay. These three different sets all yielded concordant and satisfactory results.

The *PMP22* gene dosages determined by the MP assay were analyzed by both DHPLC and capillary electrophoresis systems, with concordant results. Capillary electrophoresis is one of the most recent develop-

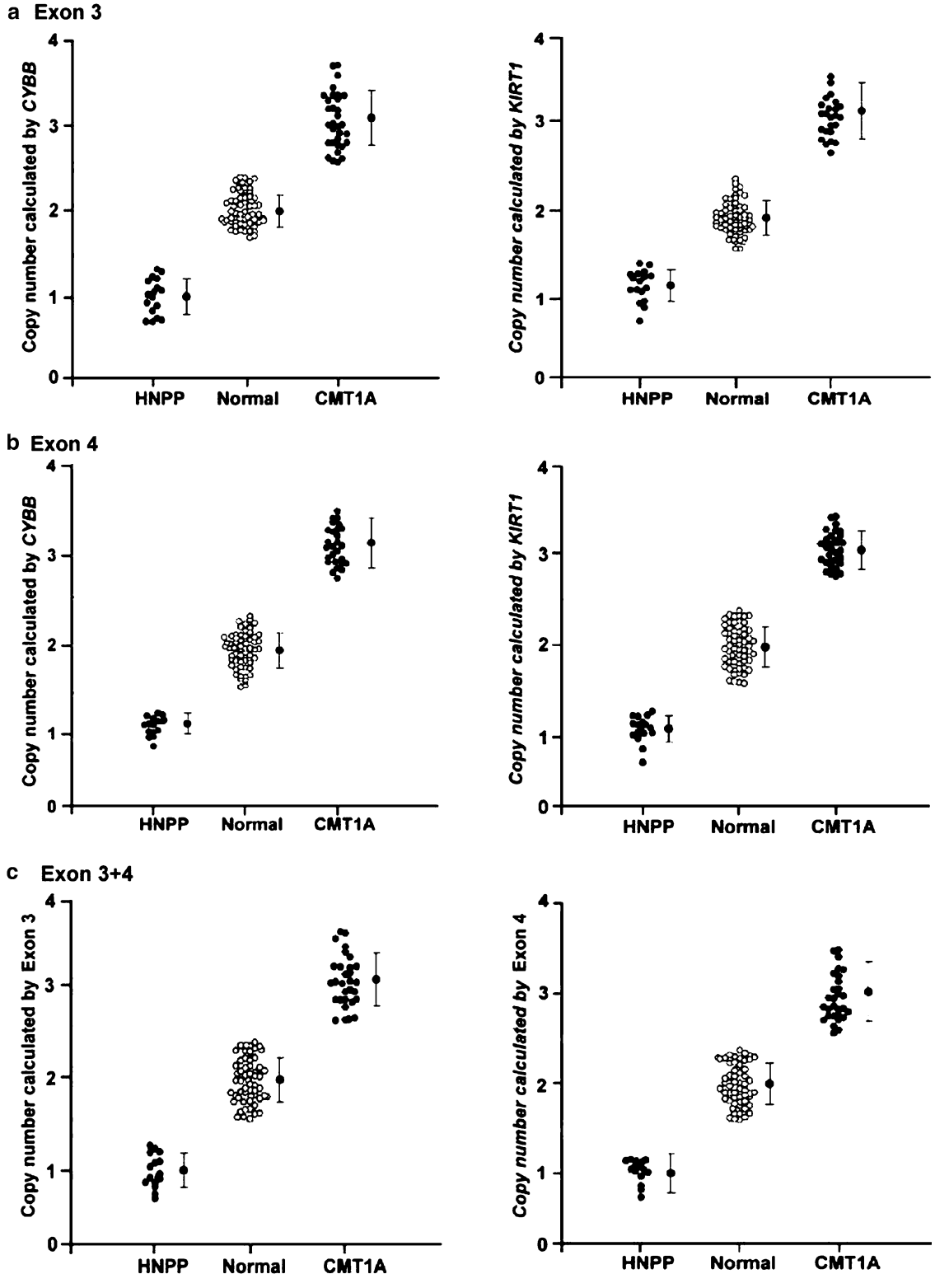

Fig. 4a–c Scatter plot of DHPLC results for CMT1A, HNPP, and normal controls; the ratio values lie in non-overlapping groups corresponding to the presence of one, two, or three copies of the *PMP22* gene, respectively. **a** Results measured by MP Set 1 (*KRIT1*/*CYBB*/exon 3 of *PMP22* gene). **b** Results measured by MP Set 2 (*KRIT1*/*CYBB*/exon 4 of *PMP22* gene). **c** Results measured by MP Set 3 (*KRIT1*/exon 3 of *PMP22* gene/exon 4 of *PMP22* gene). In **a** and **b**, copy numbers are calculated using the *CYBB* gene as genomic reference in the plots on the left, and using the *KRIT1* gene as genomic reference in the plots on the right. In **c**, the copy numbers are calculated using exon 3 of the *PMP22* gene in the left plot and by exon 4 of *PMP22* gene in the right plot (*KRIT1* gene is as genomic reference). ⟊ Mean ±SD

Table 1 Statistical evaluation of the *PMP22* gene copy numbers measured by denaturing high-performance liquid chromatography (DHPLC) vs capillary electrophoresis. For each sample class, the expected *PMP22* gene copy number, and the mean, standard deviation (*SD*), and coefficient of variation (*CV*) of DHPLC and capillary electrophoresis (*CE*) measurements are shown. *CMT1A* Charcot-Marie-Tooth disease type 1A, *HNPP* hereditary neuropathy with liability to pressure palsies

Samples	Expected copy number	Measured copy number by DHPLC (mean)	SD	CV (%)	Measured copy number by CE (mean)	SD	CV (%)
Normal ($n=61$)	2	2.02	0.19	4.99	2.01	0.25	8.54
HNPP ($n=17$)	1	1.12	0.18	9.49	1.08	0.21	12.72
CMT1A ($n=32$)	3	2.94	0.29	10.31	2.94	0.30	11.54

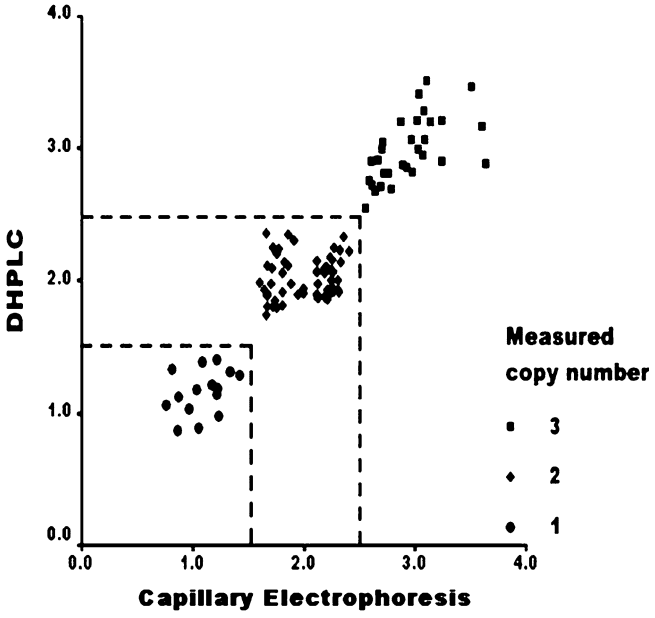

Fig. 5 Correlation of measured *PMP22* gene copy number distribution in the DHPLC system with that in the capillary electrophoresis system

ments in the detection of gene dosage (Beckmann and Schroder 2000; Latour et al. 2001; Poropat and Nicholson 1998; Young et al. 1998). This method is automatic, accurate, and fast; however, it requires fluorescently labeled primer pairs, and the post-processing of PCR products, which requires HiDi formamide for denaturation for 5 min at 95°C, followed by the use of specific sample tubes or plates to carry out the analysis. DHPLC has been used previously to detect point mutations and appears to be an economical and highly sensitive method of mutation detection. Here, we report a new application of this system that is sensitive enough to detect changes in gene copy number from one to two to three copies of the *PMP22* gene. Compared with capillary electrophoresis, DHPLC does not require fluorescence labeling of the PCR primers, and unpurified PCR products can be used. This method required only 2 h for PCR and about 30 min of DHPLC to analysis all three MP sets. The average cost of DHPLC analysis is less than US$ 3 per sample. All the above show that this method is very well suited to diagnosing a duplication causing CMT1A or a deletion causing HNPP.

Dehainault et al. (2004) described an application to detect gene rearrangements of the *RB1* gene using MP and DHPLC. Their report used the same principles as MP/DHPLC to determine relative gene dosage. They used fluorescence to detect gene dosage, thereby increasing sensitivity by ~100-fold compared with UV detection. In our study, we introduced a reliable method using DHPLC with non-fluorescent detection to quantify *PMP22* gene copy number, which is more cost-effective with same resolution. Furthermore, Dehainault et al. (2004) improved the amplification of high GC content DNA using a touchdown protocol. The principles of the touchdown protocol raise a doubt about equitable competition and the results may not be a very precise quantification of gene dosage. In our previous work, we have successfully demonstrated the application of DHPLC to gene dosage analysis of the *SMN1*/*SMN2* genes of spinal muscular atrophy. However, amplification of DNA sequences with high GC content is also a challenge in this study and requires a lot of time to choose the appropriate primer combination for our competitive PCR protocols.

We have demonstrated that MP coupled with DHPLC can accurately distinguish two gene copies from one and three copies, and proved that it is a rapid, reliable, low-cost system for the genetic diagnosis of duplication and deletions. Thus, further exploitation of the precise measurement of gene copy number using this protocol can be expected in the future.

In conclusion, MP/DHPLC analysis permits high sample throughput and can be used for large-scale screening projects. This method is a rapid, reliable, low-cost, and non-fluorescence-based test system for the genetic diagnosis of the CMT1A duplication and the HNPP deletion. It can be used as an alternative to previous methods for detection of these genetic events and can also be extended to the measurement of any copy number defects that are associated with a genetic disease.

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