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MLPA Method for *PMP22* Gene Analysis

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Abstract

DNA copy number alterations are responsible for several categories of human diseases and syndromes. These changes can be detected by cytogenetic studies when there is involvement of several kilobases or megabases of DNA. Examination of sub-microscopic changes is possible by using short probes flanked by the same primer pairs. Multiplex ligation-dependent probe amplification (MLPA) is a simple, high resolution method by which not sample nucleic acids but probes added to the samples are amplified and quantified. Charcot- Marie-Tooth disease type 1A (CMT1A) is the most common inherited peripheral neuropathy with a prevalence of about 1/10000. By a gene dosage mechanism, commonly trisomic overexpression of *PMP22* results in CMT1A whereas its monosomic underexpression causes hereditary neuropathy with liability to pressure palsies (HNPP). We applied MLPA to the *PMP22* gene in order to develop an efficient and sensitive test for detecting these gene dosage changes. The method was used on 56 samples collected for a previous comparative study on routine methods for CMT1A diagnosis used in our laboratory. All diagnoses agreed with results from other methods. The MLPA *PMP22* assay is a simple, fast and accurate screening test for molecular diagnosis of CMT1A and HNPP.

Key words: DNA, method MLPA, CMT1A duplication, HNPP deletion

Introduction

Charcot-Marie-Tooth disease (CMT) is a group of genetic peripheral neuropathies characterised by a combination of progressive muscle weakness and atrophy with a distally pronounced sensory dysfunction.[1] The phenotypic expression of the disease is heterogeneous.[2] Approximately 80% of CMT patients show a predominantly demyelinating peripheral neuropathy and are classified as CMT1A.[3] By far the most often observed genetic defect in this group is an intrachromosomal duplication of a 1.5 Mb DNA fragment on chromosome 17p12[4,5] harbouring the gene encoding *PMP22*. In some cases, point mutations in *PMP22* have also been found to be associated with peripheral neuropathies.[3] In general, *PMP22* point mutations appear to cause a more severe phenotype but both, *PMP22* duplication and *PMP22* point mutation-based neuropathies, are classified as CMT1A. Deletion of the PMP22 gene, due to the reciprocal deletion of the duplicated chromosomal segment in CMT1A described above, leads to a mild variant of a peripheral neuropathy named hereditary neuropathy with liability to pressure palsies (HNPP).[6]

There is a wide range of approaches for molecular diagnosis of CMT1A and HNPP. Most established techniques rely on dosage analysis, using either DNA probes which detect Restriction Fragment Length Polymorphisms (RFLPs)[5,7] or more recently, PCR based microsatellite markers from within the duplicated region.[8] The use of fluorescent *in situ* hybridisation (FISH) to directly visualise the number of *PMP22* gene copies on interphase chromosomes[9] has also been documented.

Alternative methods rely on the detection of the novel junction fragments produced following unequal crossing-over. Pulsed field gel electrophoresis (PFGE) can be used to examine the entire 1.5 Mb region.[10] Further definition of the hotspot region revealed proximal and distal CMT1A-REP specific restriction sites leading to the development of Southern blot probes, and more recently polymerase chain reaction (PCR) based tests.[11,12] Due to the nonlinear nature of the amplification reaction, PCR techniques are not inherently quantitative, but several modifications have been made to obtain quantitative information from PCR based assays. One such modification is locus-specific PCR amplification where the PCR products were cut by a restriction enzyme and visualizing in agarose gel, giving a clear and specific pattern for CMT1A patients who have a recombinant CMT1A-REP.[13] On the other hand real-time PCR method is well suited to semi-automated diagnostic applications where relatively small loci number need to be assessed. In real-time analysis, because data are collected throughout the reaction, the optimal stage for analysis can be decided after examination of amplification profiles. Experiments are performed in a thermal cycler that incorporates

an optical system for excitation of fluorochromes and monitoring of emitted wave lengths of light. The accumulation of PCR product is monitored by staining using an intercalating dye or by dual-labelled probes.

With the currently available technology, molecular genetic diagnosis still remains a labour intensive and costly procedure. We therefore applied multiplex ligation-dependent probe amplification (MLPA) to DNA based measurement of copy number at *PMP22*. MLPA identifies the target sequence by hybridisation of two adjacent complementary probes.[14] The probes are subsequently joined by a ligation reaction and copy sequences are amplified in a multiplex PCR reaction using unique primers attached to the probes (Figure 1). Only probes hybridised to a target sequence will be ligated and subsequently amplified in the PCR reaction. After separation by capillary gel electrophoresis, the amplification product can be analysed. The peak area of each amplification product reflects the relative copy number of that target sequence, and sequences located at chromosome ends that are deleted or duplicated can be easily identified. Compared to other techniques, an MLPA reaction is fast, cheap and very simple to perform. The equipment required is present in most molecular biology laboratories: thermocycler with heated lid and capillary electrophoresis equipment. With MLPA, it is possible to perform a multiplex PCR reaction in which up to 45 specific sequences are simultaneously quantified. Amplification products are separated by sequence type electrophoresis. As only one pair of PCR primers is used, MLPA reactions result in a very reproducible gel pattern with fragments ranging from 130 to 490 bp. Moreover, MLPA reactions require a minimum of only 20 ng human DNA.

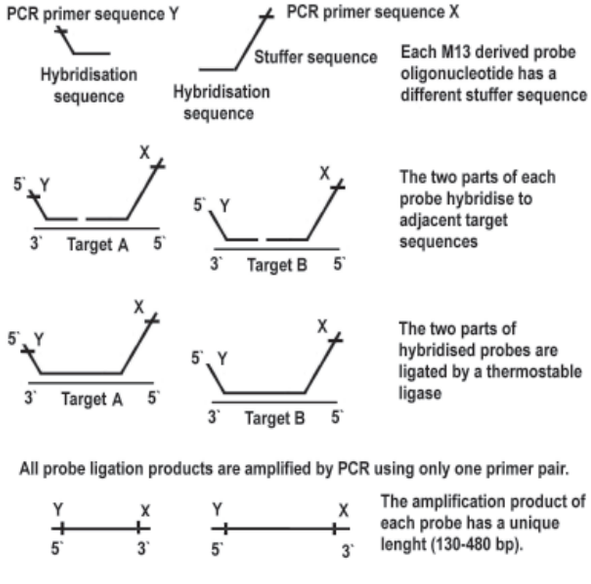

Figure 1. Principle of MLPA.

The advantages of MLPA include high resolution, high multiplicity (up to 45 loci at a time) and high sensitivity, high throughput (up to 96 samples per experiment) and low investment. In this study, we applied MLPA to the *PMP22* gene for determining DNA dosage in CMT1A/HNPP patients.

Experimental

PMP22 probe set

MLPA SALSA P033 CMT kit was supplied from Microbiology Research Centre Holland (MRC-Holland). Content of a kit is following: SALSA Probe-mix (mix of 37 probes: 8 probes are within the *PMP22* region that is duplicated in CMT1A disease and deleted in HNPP and remain 29 probes are control probes), SALSA MLPA buffer, Ligase-65, Ligase-65 Buffer A, Ligase-65 Buffer B, SALSA PCR buffer, SALSA PCR Primer mix (mix of two PCR primers + dNTPs), SALSA Polymerase and SALSA Enzyme dilution buffer. All kit constituents are stable when stored at –15 to –25 °C.

Apparatus

The CEQ 8000 Genetic Analysis System is a fully automated system capable of determining the base sequence and fragment length of DNA samples that have been prepared with Beckman Coulter dye-labelled reagents. Colour, dye-labelled terminator chemistry kits are used to process samples for base sequence analysis. Generation of samples for fragment length analysis is performed using dye-labelled primers. The CEQ 8000 accepts up to 96 colour samples in a microplate. Each row of eight samples (sample set), containing labelled DNA fragments, is automatically denatured and then separated by capillary electrophoresis. The replaceable medium (separation gel) is automatically replaced in the eight capillaries after each separation. The separation gel supply is an easily replaced cartridge with a capacity sufficient for a full microplate (96 samples). Detection is by laser-induced fluorescence in spectral channel. The channel raw data sets generated by each of the eight capillaries are automatically processed to produce high quality base sequences or fragment lists after separation. Raw and analyzed data are stored in a database and may also be exported in file compatible with common analysis applications. The hardware carries out the task of sample handling as well as tasks associated with the separation and detection phases of electrophoresis.

MLPA analysis

DNA samples were diluted with TE to 5 μL and were heated at 98 °C for 5 minutes in 200 μL tubes in a thermocycler with a heated lid (eppendorf Mastercycler personal). After addition of 1.5 μL SALSA Probe-mix mixed with 1.5 μL MLPA buffer, samples were heated

for 1 minute at 95 °C and then incubated for 16 hours at 60 °C. Ligation of annealed probes was performed by diluting the samples to 40 μL with Ligase-65 dilution mix containing Ligase-65 enzyme, and incubation for 15 minutes at 54 °C. The ligase enzyme was inactivated by heating at 98 °C for 5 minutes and ligation products were amplified by PCR. 10 μL of the ligation reaction was added to 30 μL PCR buffer. While at 60 °C, 10 μL of a buffered solution containing the SALSA PCR-primers, SALSA Enzyme Dilution buffer and SALSA Polymerase were added. PCR was for 33 cycles (30 seconds at 95 °C, 30 seconds at 60 °C and 60 seconds at 72 °C). Samples amplified with one D4-labelled and one unlabeled primer were analysed on a Beckman CEQ 8000 capillary electrophoresis system.

Data-Analysis

Excel software was used to record peak areas corresponding to the signal from each probe. Each signal of CMT1A probe mix was determined and copied to the Excel worksheet. In the next steep the total peak surface of all peaks was determined for each sample. To become independent of the amount of input DNA per samples (amount of signal), the peak fractions were calculated with the equation:

$$F(a) = A(a)/S \qquad (1)$$

where *a* represents individual peak, *A(a)* is surface of individual peak and *S* is the total peak surface of all peaks.

For the normalization, the "normal" peak fractions need to be calculated. For this purpose, control sample is used. For the control sample the average peak fractions were calculated by the formula:

$$N(a) = A(a)/S \qquad (2)$$

Finally, the normalised fractions F_{nor} were calculated:

$$F_{nor}(a) = F(a)/N(a) \qquad (3)$$

Expected normalised values are 1.0 in the absence of copy number change, and 0.5 and 1.5 in the case of heterozygous deletion and duplication, respectively.

Quality control

According to the CEQ8000 Reference Guide, best results are obtained if the fluorescent signals range between about 15000 and 150000 relative fluorescent units (RFU). Our experiments showed that reproducible results are obtained when all peak heights are more than 5000 RFU. However, peaks were considered unreliable if they were outside predefined thresholds.

Table 1. Samples Screened.

Sample	Method(s)[a]	Mutation Found[b] by	
		FISH and/or PCR	MAPH
S1	1, 2	nm	nm
S2	1, 2	nm	nm
S3	1, 2	Del	Del
S4	1, 2	Dup	Dup
S5	1, 2	nm	nm
S6	1, 2	Del	Del
S7	1, 2	nm	nm
S8	2	nm	nm
S9	1, 2	Dup	Dup
S10	1, 2	Del	Del
S11	1, 2	nm	nm
S12	1, 2	nm	nm
S13	1, 2	nm	nm
S14	1, 2	nm	nm
S15	1, 2	Del	Del
S16	1, 2	nm	nm
S17	1, 2	nm	nm
S18	1, 2	Del	Del
S19	1, 2	Del	Del
S20	1, 2	Dup	Dup
S21	1, 2	nm	nm
S22	1, 2	nm	nm
S23	1, 2	Del	Del
S24	1, 2	nm	nm
S25	1, 2	Del	Del
S26	1, 2	nm	nm
S27	1, 2	nm	nm
S28	1, 2	nm	nm
S29	1, 2	nm	nm
S30	1, 2	nm	nm
S31	1, 2	nm	nm
S32	1, 2	nm	nm
S33	1, 2	nm	nm
S34	1, 2	nm	nm
S35	1, 2	nm	nm
S36	1, 2	nm	nm
S37	1, 2	nm	nm
S38	1, 2	Del	Del
S39	1, 2	nm	nm
S40	1, 2	Del	Del
S41	1, 2	Del	Del
S42	1, 2	nm	nm
S43	1, 2	Del	Del
S44	1, 2	Del	Del
S45	1, 2	Del	Del

[a] 1 = FISH; 2 = PCR. [b] Del = deletion; Dup = duplication; nm = no mutation find.

Table 2. Samples Screened.

Sample	Method(s)[a]	Mutation found[b] by	
		FISH and/or PCR	MAPH
S46	1, 2	Dup	Dup
S47	1, 2	Dup	Dup
S48	1, 2	nm	nm
S49	1, 2	nm	nm
S50	1, 2	Dup	Dup
S51	1, 2	Dup	Dup
S52	1, 2	nm	nm
S53	1, 2	Del	Del
S54	1, 2	nm	nm
S55	1, 2	nm	nm
S56	1, 2	Dup	Dup

[a] 1 = FISH; 2 = PCR. [b] Del = deletion; Dup = duplication; nm = no mutation find.

Results and discussion

A total of 56 samples were screened in a semiblind manner (Tables 1 and 2, Figure 2). There were a mixture of fully and partially characterized cases, as well as samples from cases in which no mutation had been found. Of the 23 mutations (8 duplications and 15 deletions) previously characterized in our laboratory with fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR), all were detected using the MLPA technique (Tables 1 and 2). One hundred percent concordance was observed for the duplications detection in CMT1A and deletions detection in HNPP on interphase nuclei with FISH and MLPA analysis of *PMP22* with capillary electrophoresis.

Using SALSA Probe-mix, samples could be categorised as: (a) normal ratios (between 0.9 and 1.18), (b) deleted ratios (<0.6 regarding probes 25–32 from *PMP22*), and (c) duplicated ratios (>1.2 regarding probes 25–32 from *PMP22*) (Figure 2). For results in uncertain ranges (0.6–0.9 and 1.18–1.2), the MLPA test could be repeated. If samples are tested in duplicate, and the average of two measures has a normal distribution with a mean of 1 and a standard deviation 0.1, the predicted rate of false positive duplications and deletions is less than 0.001%, and the predicted incidence of normal samples falling into the uncertain ranges is about 0.04%.

Among the 56 samples tested, the average of SALSA Probe-mix values in the group of 33 normal samples had a mean ratio of 1.021 and a standard deviation of 0.101 (Figure 3). This mean ratio is in range of the expected value 1 and has proved that MLPA technique is reproducible and specific method.

The MLPA approach's primary advantages over FISH and PCR are the relative simplicity and speed.

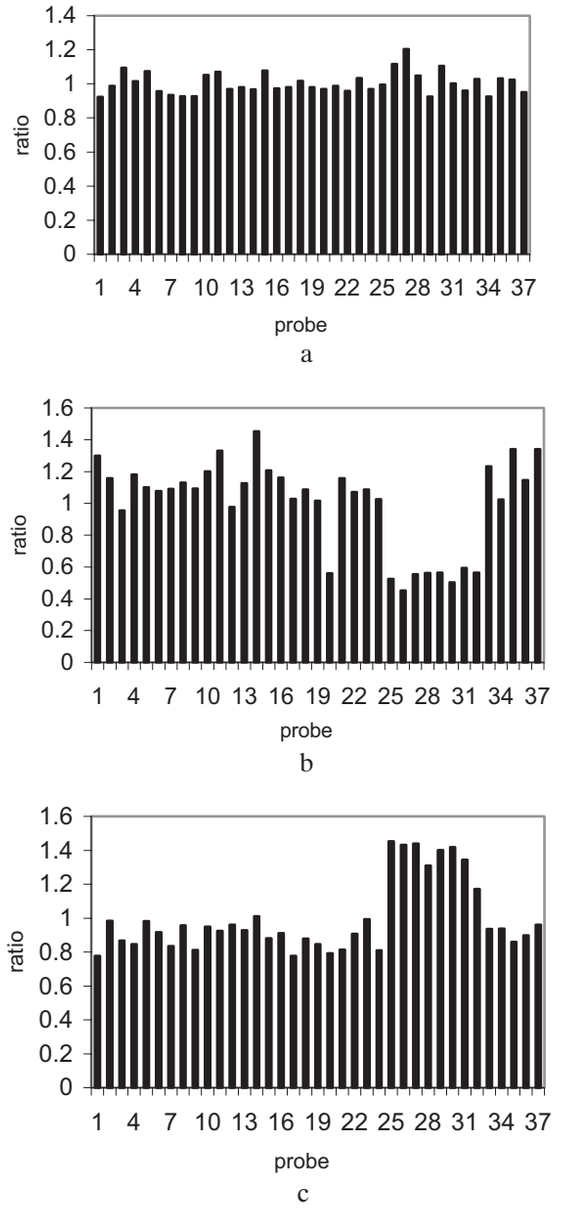

Figure 2. Analysis of different samples. a) Sample without mutation, with SD 0.06. b) Sample with deleted probes 25–32 and SD 0.05. c) Sample with duplicated probes 25–32 and SD 0.09.

Although 80% of the duplications can be detected using PCR, the breakpoints are often not determined, and rare mutations outside the hotspots will be missed. In previously published reports on MLPA,[15,16,17] recovered probes were radioactively labelled and were separated on a polyacrylamide gel. For speed and convenience, we chose to use a combination of fluorescent labelling and capillary electrophoresis. Capillary electrophoresis is becoming more widely used in mutation detection, since it provides greater sensitivity and has high-throughput capabilities.[14] We used the CEQ 8000 (Applied Beckman coulter), which allows the simultaneous analysis of 8 samples.

In contrast to many other methods, this technique should be easy to implement in a standard diagnostic laboratory, since no new technology needs to be introduced. The critical phases are hybridisation and PCR, and the products can be analysed on any apparatus that is used for sequence analysis. Furthermore, it can easily be applied to any (disease) gene of interest, and the resolution provided and the potential of array implementation may even allow future genomewide screening.

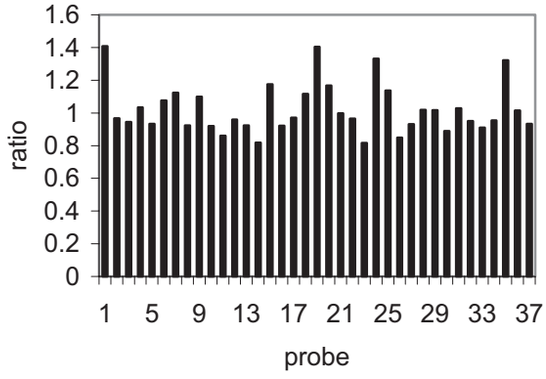

Figure 3. Average of 33 normal samples with a mean of 1.021 and a standard deviation of 0.101.

Polymorphisms or single base mutations in the probe binding regions may affect MLPA results. The short length of the specific probe region in the MLPA probes means that nucleotide mismatches at the probe binding site may influence probe hybridisation, prevent ligation and affect detection, so that single base changes may appear as exon deletions.[13] For this reason, we recommend that all simple fragment deletions found by MLPA be confirmed by an independent method, and that sequencing of the target region be undertaken should an independent test not indicate deletion. The power of MLPA detection will be also weakened by mosaicism; for example, a *PMP22* duplication present in only 20% of the cells analysed would go undetected by MLPA, as it would by many other methods relying on DNA dosage to detect deletions and duplications.[18]

Conclusions

We have presented here the MLPA technique that has proved to be a simple, fast, sufficiently sensitive and sequence specific test for molecular diagnosis of CMT1A and HNPP. In comparison to DNA arrays, MLPA is cheap, more sensitive and requires much less sample preparation time. The equipment needed is present in many molecular biology laboratories. Although limited to 37 target sequences/reaction, this is sufficient for many routine tests in which only a specific question needs to be solved.

Acknowledgements

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Povzetek

Pri ljudeh so spremembe v številu kopij DNA odgovorne za mnoge vrste bolezni in sindromov. Te spremembe se lahko odkrijejo s citogenetskimi raziskavami kadar imamo prisotnih nekaj kilobaz ali megabaz DNA. Preiskovanje sub-mikroskopskih sprememb je možno ob uporabi kratkih prob sestavljenih z enim parom prajmerjev. Hkratno pomnoževanje od ligacije odvisnih sond (MLPA) je enostavna metoda visoke ločljivosti, pri kateri se ne pomnožujejo in določajo nukleinske kisline vzorcev, ampak probe dodane k vzorcem. Charcot-Marie-Tooth-ovo obolenje tipa 1A (CMT1A) je najpogostejše dedno živčno mišično obolenje z razširjenostjo 1/10000. Pri CMT1A je prisoten presežek gena *PMP22*, medtem ko je njegov primanklaj značilen za HNPP. V ta namen, smo v okviru gena *PMP22* uporabili MLPA metodo, da bi razvili čimbolj učinkovit in občutljiv test za odkrivanje njegovih sprememb. Uporabili smo 56 vzorcev predhodno testiranih z obstoječimi rutinskimi metodami laboratorija v okviru CMT1A diagnostike. Dobljeni rezultati so se ujemali z rezultati preostalih metod, kar pomeni, da je MLPA *PMP22* analiza zanesljiva in v primerjavi z drugimi metodami precej enostavna in hitra v okviru molekularne diagnostike CMT1A in HNPP.