Advantages of ddPCR in detection of *PLP1* duplications

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Summary	**Pelizaeus–Merzbacher disease (PMD) is an X-linked, recessively inherited disorder associated with hypomyelination in the brain white matter. Mutations involving the proteolipid protein 1 gene (*PLP1*) located on Xq22.2 are responsible for PMD. *PLP1* duplication is the major genetic abnormality in PMD patients. In this study, we utilized droplet-digital polymerase chain reaction (ddPCR) as a potential method to detect *PLP1* duplications. Samples from four PMD patients and one of their mothers were used as positive controls. They had been previously diagnosed as having an additional *PLP1* copy by chromosomal microarray testing. Genomic copy number of *PLP1* was analyzed in triplicate experiments and compared with reference genes *XIST* and *AR* on the X-chromosome, and *RPP30* and *RPPH1* on the autosomes. As a result, precise results were obtained for each triplicate procedure. Thus, we concluded that triplicate experiments are no longer necessary. Compared to other methods, including fluorescence *in-situ* hybridization, multiplex ligation-dependent probe amplification, chromosomal microarray testing, and quantitative PCR, we were able to establish ddPCR results rapidly with very small amounts of DNA. In conclusion, we showed that ddPCR can be a potential diagnostic tool to confirm genomic copy number as a routine clinical application, including in prenatal diagnostic settings.**

Keywords: PLP1 duplications, droplet digital polymerase chain reaction, copy number variations

1. Introduction

Pelizaeus–Merzbacher disease (PMD) is a genetic disorder associated with hypomyelination in brain white matter, and most patients with PMD exhibit motor developmental delay, hypotonia, horizontal nystagmus, and progressive spasticity (*1*). The proteolipid protein 1 gene (*PLP1*) located on Xq22.2 is responsible for PMD. Thus, PMD is recognized as an X-linked recessive disorder and most patients are male. Their *PLP1* abnormalities are often inherited from their carrier mothers. It is also known that two-thirds of PMD patients generally show chromosomal microduplications involving the Xq22.2 region, which

harbors *PLP1* (*2*). For these reasons, it is recommended to first screen for *PLP1* duplication in cases of patients suspected of having PMD. In such cases, fluorescence in-situ hybridization (FISH) and multiplex ligation-dependent probe amplification (MLPA) methods have been used previously. The more efficient method is chromosomal microarray testing, because this method can detect not only duplications but also identify extent of duplications.

When we detected *PLP1* duplications in probands, diagnosis of carrier status of their mothers is often required. Furthermore, when mothers carry *PLP1* duplications, prenatal diagnosis is often required for subsequent pregnancies. In such cases, analytical methods need to fulfill certain conditions. Results should be rapidly and precisely obtained. It would be advantageous if the amounts of DNA required for these tests are small. Consequently, chromosomal microarray testing has limitations for the purpose of this screening.

The aim of this study was to establish a simple and rapid detection method to confirm *PLP1* duplications. With this aim, we utilized droplet-digital polymerase

chain reaction (ddPCR) as a potential method as a 'proof-of-concept'.

2. Materials and Methods

2.1. *Materials*

Positive control samples were prepared as described in previous studies (*3*). Four PMD patients and one carrier mother were used. All patients had already been diagnosed to have *PLP1* duplications by chromosomal microarray testing. The sizes of the duplicated regions are shown in Table 1. Control samples from 1 normal male and 1 normal female were also used.

This study was performed in accordance with the Declaration of Helsinki and approved by the ethics committee of the Tokyo Women's Medical University. Written informed consent was obtained from patients and their parents before peripheral blood samples were acquired.

2.2. *Methods*

By use of the QIAamp DNA extraction kit (QIAGEN, Hilden, Germany), genomic DNA was extracted from peripheral blood samples. DNA concentration was calculated using a Qubit®2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). *PLP1* was the target in this study. X inactive specific transcript (*XIST*), androgen receptor (*AR*), ribonuclease P/MRP 30kDa subunit (*RPP30*) and ribonuclease P RNA component H1 (*RPPH1*) genes were selected as references. *XIST* (Xq13) and *AR* (Xq12), were used as references for the X-chromosome. *RPP30* (10q23) and *RPPH1* (14q11) were used as reference for the autosomal chromosomes, as they are located in conserved regions, known to have low frequency of copy number variants (CNVs) (*4,5*).

Our priority in this study was to confirm the simplicity and accuracy of ddPCR. Because we used an intercalation system rather than TaqMan® hydrolysis probes, primers were not labeled with fluorescent dyes. We designed the primers with Primer3Plus (*https://primer3plus.com/*) and checked for homology using the UCSC Genomic Browser BLAT (*https://genome.ucsc.edu/cgi-bin/hgBlat*). Primers details are given in Table 2.

The ddPCR reaction mixtures were prepared as 20 μL total volumes, which included 10 μL 2 × QX200 ddPCR EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA), 1 μL each of the forward and reverse primers (1 μM), 1 μL DNA (20 ng/μL), and 7 μL RNase/DNase-free water. We loaded 20 μL of reaction mixture and 70 μL of QX200 Droplet Generation Oil for EvaGreen (BioRad Laboratories) into a QX100/200 DG cartridge (Bio-Rad Laboratories), which was transferred into a QX200 Droplet Generator (Bio-Rad Laboratories). Droplets containing 40 μL of oil and sample emulsion were transferred into clean 96-well

Table 1. Summary of the samples

Samples	Duplication sizes
Patient 1	648-Kb
Patient 2	603-kb
Patient 3	656-kb
Patient 4	948-kb
Carrier mother	603-kb
Control male	None
Control female	None

PCR plates. To avoid contamination and evaporation, the plates were sealed using a PX1 PCR plate sealer (Bio-Rad Laboratories) with pierceable foil heat seals (Bio-Rad Laboratories). Then, PCR was performed with a Gene Amp PCR system 9700 (Thermo Fisher Scientific, Waltham, MA, USA). The thermal cycling conditions were as follows: 95℃ for 5 min (1 cycle); then 40 cycles of 95℃ for 30 s and 60℃ for 1 min; 4 ℃ for 5 min, 90℃ for 5 min, and then held indefinitely at 4℃. The ramp rate was 2℃/sec in all steps. After thermal cycling, droplets were analyzed for positive and negative signals using the QX200 droplet reader (Bio-Rad Laboratories). For each ddPCR sample, the same process was performed in triplicate. Data analysis was performed when the number of droplets produced was more than 10,000.

For data analysis, QuantaSoft Version1.7.4 software (Bio-Rad Laboratories) was used to statistically analyze the obtained data. Genomic copy number was calculated using the reference genes as follows: genomic copy number = (A/B) × C, where A is the concentration of the target DNA, B is the concentration of the reference DNA, and C is the number of copies of the reference gene. Because *RPPH1* and *RPP30* are located on the autosomal chromosomes, both are defined to have two copies. Copy numbers of *AR*, *XIST* and *PLP1* on the X-chromosome were different between males and females. Normal males and females are defined to have one and two copies, respectively.

3. Results and Discussion

Copy numbers of *AR*, *XIST*, and *PLP1* were calculated with two reference genes, *RPP30* and *RPPH1*, and obtained results were summarized in Table 3. Copy numbers of *RPP30* and *RPPH1* were calculated for each other as a reference. As shown, all replicates showed the copy numbers of *RPP30* and *RPPH1* close to "2", because these two genes are located on the autosomal chromosomes. In comparison, copy numbers of *AR* and *XIST* were different between males and females. Males and females showed the copy numbers of them as "1" and "2", respectively. It is reasonable because *AR* and *XIST* are located on the X-chromosome. From these findings, it was confirmed that ddPCR system can be used for detection of genomic copy number accurately.

In this study, *PLP1* duplications were targeted. Final

Table 2. Designs of the used primers

Targeted genes	Sense primers	Antisense primers	Product length (bp)
PLP1	5'-TCACAACCCCAAAGCAGCACATTTC-3'	5'-CGGCTAATTCAAAATCCAGCAAAGGG-3'	417
IRAK1	5'-AGCTCTGCATCATCGTCGT-3'	5'-CCAGCTTCTGGACCATCTTC-3'	76
XIST	5'-TGAGACCTGAGGACTGCAAA-3'	5'-AGCTTGGCCAGATTCTCAAA-3'	77
AR	5'-CCAGCAGAAATGATTGCACTA-3'	5'-CATTTCCGAAGACGACAAGA-3'	70
RPP30	5'-GATTTGGACCTGCGAGCG-3'	5'-GCGGCTGTCTCCACAAGT-3'	62
RPPH1	5'-GTCAGACTGGGCAGGAGATG-3'	5'-TGGCCGTGAGTCTGTTCC-3'	75

Table 3. Results of ddPCR

Samples		Versus RPPH1				Versus RPP30			
		AR	*XIST*	*RPP30*	*PLP1*	*AR*	*XIST*	*RPP30*	*PLP1*
Patient 1	1	1.08	1.10	2.14	1.84	1.00	1.04	1.86	1.72
	2	1.10	1.06	2.18	1.92	1.00	0.96	1.84	1.76
	3	1.04	1.02	2.08	1.82	1.00	0.98	1.72	1.76
Average		1.07	1.06	2.13	1.86	1.00	0.99	1.81	1.75
SD		0.02	0.02	0.03	0.03	0.00	0.02	0.04	0.01
Patient 2	1	1.22	1.02	2.20	2.04	1.10	0.94	1.82	1.86
	2	1.16	1.00	2.18	2.02	1.04	0.98	1.80	1.80
	3	1.20	1.14	2.32	2.10	1.02	0.98	1.72	1.88
Average		1.19	1.05	2.23	2.05	1.05	0.97	1.78	1.85
SD		0.02	0.04	0.04	0.02	0.02	0.01	0.03	0.02
Patient 3	1	1.02	0.98	2.02	1.76	1.02	0.98	1.98	1.74
	2	1.06	1.08	2.16	2.02	1.00	1.00	1.86	1.88
	3	1.14	1.02	2.14	1.98	1.06	0.96	1.86	1.84
Average		1.07	1.03	2.11	1.92	1.03	0.98	1.90	1.82
SD		0.04	0.03	0.04	0.08	0.02	0.01	0.04	0.04
Patient 4	1	1.10	1.14	2.12	2.02	1.04	1.08	1.88	1.90
	2	1.04	1.08	2.14	2.04	0.96	1.00	1.86	1.90
	3	1.04	1.06	2.16	1.90	0.98	0.98	1.86	1.76
Average		1.06	1.09	2.14	1.99	0.99	1.02	1.87	1.85
SD		0.02	0.02	0.01	0.04	0.02	0.03	0.01	0.05
Carrier	1	2.14	2.06	2.14	2.96	2.02	1.94	1.88	2.78
mother	2	2.08	2.04	2.08	3.00	2.00	1.96	1.92	2.88
	3	1.96	2.06	1.98	2.94	1.98	1.96	2.02	2.98
Average		2.06	2.05	2.07	2.97	2.00	1.95	1.94	2.88
SD		0.05	0.01	0.05	0.02	0.01	0.01	0.04	0.06
Male	1	1.00	0.92	2.06	1.00	0.96	0.88	1.86	0.96
control	2	1.04	1.04	2.18	1.00	0.96	0.96	1.84	0.92
	3	1.00	0.94	1.94	0.96	1.02	0.98	2.08	1.00
Average		1.01	0.97	2.06	0.99	0.98	0.94	1.93	0.96
SD		0.01	0.04	0.07	0.01	0.02	0.03	0.08	0.02
Female	1	2.06	2.04	2.02	1.96	2.04	2.02	1.98	1.94
control	2	2.08	2.10	2.10	2.00	1.96	1.98	1.90	1.90
	3	2.16	2.02	2.18	2.00	1.96	1.94	1.94	1.94
Average		2.10	2.05	2.10	1.99	1.99	1.98	1.94	1.93
SD		0.03	0.02	0.05	0.01	0.03	0.02	0.02	0.01

SD, standard deviation.

results are graphically summarized in Figure 1. As shown, all four male PMD patients demonstrated two copies of *PLP1* and the carrier females demonstrated three copies of *PLP1*. All triplicates showed similar results (Table 3), and there were no data which showed dispersed values. From these findings, each independent ddPCR result provided precise results.

There are several diagnostic methods to detect CNVs such as FISH, MLPA, chromosomal microarray testing, qPCR, and dPCR (*6*). At first, the capacity for precise detection of *PLP1* duplications should be considered. Previously, FISH has been used to detect

PLP1 duplications. In the case of FISH, detected signals are the result of hybridization of labelled DNA probes to corresponding chromosomal regions. Thus, detected signal numbers in a set of karyotype correspond to copy numbers. However, when the targeted regions are small, it is sometimes difficult to detect duplications on the same chromosome, because two signals cannot be separately detected or resolved (*3*). In this regard, interpretations of FISH results are often subjective.

Reaction conditions for ddPCR may be similar to qPCR. Traditional qPCR, based on relative quantification of target DNA, also can be used to detect
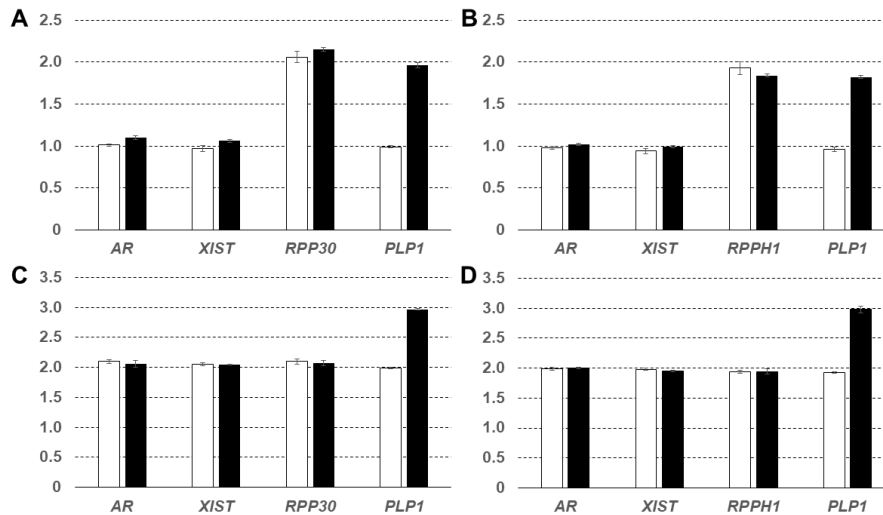
Figure 1. Graphical presentations of droplet digital PCR (ddPCR) results. Average copy number of the triplicates of the targeted genes versus reference genes are shown. Results of male patients (black; *n* = 4) and a normal male control (white; *n* = 1) were compared with *RPPH1* **(A)** and *RPP30* **(B)**. Results of a carrier female (white; *n* = 1) and a normal female control (black; *n* = 1) were also compared with *RPPH1* **(C)** and *RPP30* **(D)**. The Y-axis represents copy number estimated by ddPCR. Error bars indicate ± 2SD.

genomic copy number. Because qPCR is based on PCR amplification in which DNA is doubled in each amplification cycle, it is usually difficult to detect copy number gains of 1.5 ×. To compensate for unstable detection, independent triplicate experimental data sets are required for qPCR (*7,8*).

In comparison, we should be able to precisely detect small duplications by use of new technologies including MLPA and chromosomal microarray testing. In this study, ddPCR also provided precise results of *PLP1* duplications in PMD patients and the carrier mother. For this reason, ddPCR can be used in the same way as same as MLPA and chromosomal microarray testing.

The ddPCR method is a new technology that is based on partitioning template DNA and performing multiple independent PCR amplifications (*9*). In particular, the ddPCR system was developed to distribute template DNA randomly into emulsions of water-in-oil droplets (*10*). With data involving positive (containing target DNA) and negative (no target DNA) PCR amplifications, ddPCR provides absolute detection of genomic copy number and precise quantification of target DNA. In this study, results of all triplicates were the same for each sample. These results showed that ddPCR led to accurate CNV detection in one experimental pr°Cess, and triplicate experiments are no longer necessary.

Next, we wanted to evaluate assay time required. For FISH analysis, chromosome specimens are needed. For that, at least several days are required. Hybridization also requires time (generally, a number of days). For chromosomal microarray testing, long hybridization times are required (generally overnight). For MLPA, hybridization requires 16 hours. Thus, ddPCR has advantages with regard to required time. As

shown, ddPCR requires thermal cycling reactions and droplet read-outs. For these experiments, approximately 6 hours are needed. Compared to other methods, we were able to obtain ddPCR results rapidly.

Furthermore, we wanted to consider required sample amounts. For chromosomal microarray testing, 250 ng is required when we use the Agilent CGH Microarray system (Agilent Technologies, Santa Clara, CA, USA). This is excessive in comparison with MLPA and ddPCR. MLPA generally requires 50-200 ng. ddPCR needs only 20 ng. In this regard, ddPCR also has an advantage.

In the case of prenatal diagnosis, carrier mothers will require the result as soon as possible. Regarding sample amounts, we can only extract a small amount of DNA from amniotic fluid (generally, less than 500 ng). Therefore, the necessary time needed and sample amounts required are the critical points for prenatal diagnosis. ddPCR will fulfill these demands with precise results. In conclusion, we showed that ddPCR is a potential diagnostic tool to confirm genomic copy number as a daily clinical application, including prenatal diagnosis.

Acknowledgements

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