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ORIGINAL ARTICLE

Prenatal diagnosis of aneuploidies in amniotic fluid by multiple ligation-dependent probe amplification (MLPA) analysis

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Abstract

Prenatal diagnosis is essential in the new era of diagnosis and management of genetic diseases in obstetrics. Multiple ligation-dependent probe amplification (MLPA) is a recent technique for prenatal diagnosis for the relative quantification of 40 different nucleic acid sequences in one single reaction. We had utilized the MLPA technique in detecting aneuploidies in amniotic fluid samples from 25 pregnant women from the Obstetrics and Gynaecology Department UKMMC, versus the quantitative fluorescent polymerase chain reaction (QF-PCR) method. Conclusive results were obtained in 18 cases and all were concordant with that of the QF-PCR. All four cases of trisomies were correctly identified including one case with maternal cell contamination.

Keywords: prenatal diagnosis, PCR, amniotic fluid, multiple ligation probe amplification (MLPA), DNA

INTRODUCTION

Chromosomal abnormalities occur in approximately 1/150 life births. They are seen in 50% of first and 20% of second trimester spontaneous abortions respectively.[1] Chromosomal abnormalities, either numerical abnormalities or aneuploidies, can occur in gametes and therefore will be present in all cells of the affected individual or in a fraction of the cells during mitosis. In the latter, where there is genetic mosaic, prenatal diagnosis can be a problem, since some of the cells with chromosome abnormalities may not be available for laboratory diagnosis. Instead, individuals who are mosaic for chromosomal aneuploidies generally tend to have a less severe form of the syndrome compared to those with full trisomy, such as mild physical abnormality that might be missed during fetal ultrasound.

Prenatal diagnosis is integral in the field of obstetrics, allowing rapid reassurance for those with normal results, and earlier decision on pregnancy management in the cases with abnormalities. Screening for chromosomal abnormality is done in patients with advanced maternal age (>35 years), previous history of trisomy or abnormality on fetal ultrasound. For patients at 11-13 weeks of pregnancy, the choice of test are measurement of nuchal translucency (NT) measured by ultrasound, Pregnancy Associated Plasma Protein (PAPP-A) and free beta subunit of human chorionic gonadotrophin (β hCG). Increased thickness of NT combined with advanced maternal age has a rate of detection of 72% - 75% for Down syndrome.[2] Increased NT measurement is also associated with other chromosomal abnormalities such as Edwards and Patau syndrome.[3] A study in the first trimester screening showed 85% accuracy rate in detection of chromosomal abnormalities with 5% false positive result.[4] Second trimester screening is performed for patients at 15 - 20 weeks of pregnancy where maternal serum are tested for β-hCG, alpha fetoprotein and estriol. Typically, trisomy 21 is associated with high maternal levels of β-hCG and low levels of alpha-fetoprotein and unconjugated estriol.[2]

Multiple ligation-dependent probe ampli-

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fication (MLPA) technique is a recent and sensitive molecular technique that involves relative quantification of 40 different nucleic acid sequences in one reaction.[5] MLPA involves six processes; DNA denaturation and hybridization, ligation, PCR amplification, capillary electrophoresis and data normalization. Instead of amplifying the DNA, MLPA uses a pair of oligonucleotide probes that will hybridize to the target DNA sequence. Once hybridization occurs, these two oligonucleotide probes will be ligated and subsequently amplified by PCR. The relative signal strength of each amplification product is proportionate to the copy number of the target sequence in the sample. MLPA probes that do not find a target sequences cannot be amplified by PCR, thus no signal is detected. The different lengths of products are separated on an automated capillary sequencer and the peak areas are identified.[5-8]

QF-PCR is another fast and specific method in detecting aneuploidies.[9] It is a molecular based method performed using DNA instead of cultured fetal cells that is used in conventional karyotyping.

We studied the usefulness of MLPA technique in detecting common aneuploidies: trisomies 13, 18 and 21 and sex chromosomes aneuploidies in amniotic fluid of cases referred for amniocentesis in UKM Medical Centre (UKMMC) in comparison to the QF-PCR.

MATERIALS AND METHODS

This study was approved by the Institutional Review Board and Ethics Committee of UKMMC and written informed consent were obtained from the patients. The patients in the study were either suspected to have fetal aneuploidies from detail ultrasound scanning or had a high risk for Down syndrome based on serum screening and nuchal translucency measurement. The amniotic fluid specimens from amniocentesis performed at the Feto-Maternal Unit of the department in UKMMC were collected in 10 mls sterile containers for the MLPA and QF-PCR analyses. QF-PCR was performed in a laboratory of another institution. Pregnant women with chorioamnionitis, Rh iso-immunization and positivity for infectious diseases, for example hepatitis B and HIV positive, were excluded from the study. The control specimens consisted of blood specimens collected from normal volunteers with consent. Results from the MLPA analysis were available within 72 hours after amniocentesis and they were interpreted without the knowledge of QF-PCR.

DNA extraction

DNA extraction was done immediately or within 24-48 hours of sample collection, using QIAamp DNA Mini Kit from Qiagen, Germany according to protocol for blood and body fluid with some modifications to obtain a higher yield of DNA. DNA quantification was performed on Qubitfluorometer. The specific dye used produced fluorescence upon binding to DNA. The fluorescence signal was proportionate to the amount of DNA in the sample.

MLPA Analysis

MLPA was performed using SALSA MLPA P095 Aneuploidy kit (MRC-Holland). We had evaluated the performance of the different MLPA probes and determined the average relative probe signal and standard deviation for each probe in the samples with normal karyotype. MLPA reaction was performed using DNA thermal cycler GenAmp PCR System and the separation and quantification of the amplification products were by Capillary electrophoresis on 3130 ABI-genetic analyzer (Applied Biosystem, USA). We had used approximately 25-80 ng of DNA concentration in a total volume of 5-7 µl for analysis. The MLPA reaction was performed according to the method described by Schouten et al, 2002. The sample was heated for 5 minutes at 98^{0}C to denature the DNA, and cooled to 25^{0}C. Hybridization process was performed by adding 3 µl of mixture (1.5 µl SALSA probemix, 1.5 µl MLPA buffer) to denatured genomic DNA. The mixture was heated for 1 minute at 95^{0}C followed by 6 hours at 60^{0}C. After overnight incubation, heat stable ligase enzyme-65 was added for ligation process and the temperature was increased to 54^{0}C for 15 minutes for optimal reaction, followed by ligase inactivation at 98^{0}C for 2 minutes prior to PCR. 10 µl of this ligation mix was added to 40 µl of PCR reagent containing dNTPs, Taq polymerase and PCR primers. The reaction mixture was preheated at 95^{0}C for 1 minute, followed by 32 cycles amplification by PCR (30 seconds at 95^{0}C, 30 seconds at 60^{0}C and 60 seconds at 72^{0}C). The PCR product was separated by capillary electrophoresis using standard microsatellite analysis module for DNA fragment analysis. For capillary electrophoresis, the mixture of 1µl of PCR product, 40 µl of HI-DI formamide and 0.3 µl of Gene Scan TM 500 LIZ were denatured for 2 minutes at 95^{0}C followed by capillary analysis. For each run, two male controls were included.

Table 1: Relative probe signals in cases of trisomy 18

Chromosomes 18 probes	Relative probe signal		
	Case 1	Case 2	Case 3
TYMS probe	**0.90**	**1.26**	**1.35**
MC2R probe	**1.30**	**1.5**	**1.39**
SS18 - 1 probe	**2.06**	**1.33**	**1.22**
SS18 - 2 probe	**1.27**	**1.49**	**1.37**
SMAD4 probe	**1.55**	**1.2**	**1.15**
PMAIP1 probe	**1.53**	**1.23**	**1.25**
SERPINB2 probe	**1.28**	**1.37**	**1.51**
NFATC1 probe	**1.50**	**1.32**	**1.35**

*Note: the shaded areas show relative probe signals of > 1.3

Data analysis

Data obtained from capillary electrophoresis was analyzed using Coffalyser software version 7. In chromosomally balanced individual, all relative signals for probes was expected to be 1.0 because there are 2 copies of the target sequence in the genome. A monosomy would be indicated by a relative probe signal ≤ 0.7 and trisomy, by a relative probe signal ≥ 1.3. The diagnosis of trisomy was made using the criteria that at least 4 out of 8 chromosome specific probes have signal ≥ 1.3.[5] For the quantification of sex chromosomes, since we used male sample as controls, for normal male, the value for X and Y chromosomes probe should be close to one (1) whereas for female the value for X probe should be close to two (2) and no signal should be detected in the Y chromosome probes.[6]

RESULTS

Amniocentesis was performed within 16 weeks to 34 weeks of gestational age. The mean age of patients in this study was 30.8 years (range: 23 – 43 years). A total of 25 amniotic fluid samples were studied but there were only 18 samples that could be analyzed conclusively. Seven of the samples could not be analysed due to low quantity of DNA. From this sample, 14 samples showed normal karyotype (9 female and 5 male karyotypes).

MLPA analysis results showed four of the samples were positive for aneuploidies where three cases were positive for trisomy 18 and one sample was positive for trisomy 13. There was no case of trisomy 21 detected. The positive cases were diagnosed based on the criteria that at least four out of eight chromosome-specific probes had a relative probe signal of ≥1.3[5] (Table 1 and Table 2). There were three cases with maternal blood contamination based on direct observation of red cells contamination on amniotic samples after centrifugation of amniotic fluid. All the results were comparable with results of QF-PCR.

We determined the average relative probe signal and standard deviation for each probe in the 14 samples with normal karyotype and they showed an average relative probe signal about 1.0 (ranges from 0.939 – 1.181) (Table 3). The standard deviation from each probe ranged from 0.07 - 0.35. For precision study, intra assay co-efficient variation result ranged from 1.31% to 12.8%.

Table 2: Relative probe signals in cases of trisomy 13

Chromosomes 13 probes	Relative probe signal Case 1
BRCA2 probe	**1.39**
CCNA1 probe	**1.38**
RB1 probe	**1.37**
DLEU1 probe	**1.31**
DACH1 probe	**1.21**
ABCC4 probe	**1.36**
ING1 probe	**1.53**
ARHGEF7 probe	**1.36**

*Note: the shaded areas show relative probe signals of > 1.3

Table 3: MLPA analysis - average probe signals and precision study (intra-assay standard deviation)

MLPA probe	Chromosomal Position	PCR product size	Average probe signal	Standard deviation	Intraassay precision (Coefficient variation)
BRCA2 probe	13q12.3	355	0.988	0.135	4.45%
CCNA1 probe	13q12.3	178	1.010	0.089	5.36%
RB1 probe	13q14.3	220	1.010	0.108	3.48%
DLEU1 probe	13q14.3	400	1.013	0.246	8.85%
DACH1 probe	13q21.3	265	0.993	0.077	5.83%
ABCC4 probe	13q32	148	1.021	0.172	4.29%
ING1 probe	13q34	445	1.180	0.349	6.11%
ARHGEF7 probe	13q34	310	1.072	0.189	5.39%
TYMS probe	18p11.3	301	0.954	0.135	8.22%
MC2R probe	18p11.2	436	1.022	0.257	1.31%
SS18 - 1 probe	18q11.2	211	0.940	0.225	11.03%
SS18 - 2 probe	18q11.2	391	0.999	0.115	5.21%
SMAD4 probe	18q21.1	142	0.947	0.153	3.69%
PMAIP1 probe	18q21	172	0.966	0.112	4.32%
SERPINB2 probe	18q21.3	346	1.058	0.106	8.77%
NFATC1 probe	18q23	256	1.026	0.188	3.75%
STCH probe	21q11	247	0.939	0.115	4.54%
USP25 probe	21q11.2	202	0.909	0.142	3.70%
NCAM2 probe	21q21.1	166	0.955	0.147	2.36%
APP probe	21q21.3	337	1.104	0.099	7.58%
TIAM1 probe	21q22.1	427	1.084	0.186	7.24%
SOD1 probe	21q22.1	292	0.966	0.136	4.90%
SIM2 probe	21q22.2	136	1.010	0.358	12.84%
TFF1 probe	21q22.3	382	1.006	0.127	6.40%

DISCUSSION

Negative amplification reaction was one of the challenges we faced during MLPA reaction process. Initially, we had difficulties in getting the entire probe amplification product even though the amount of the DNA was adequate. The technical problems were due to poor mixing technique and inaccurate pipetting of the reagents and/or samples. Both the MLPA buffer and polymerase reagents used in the MLPA reaction were viscous solution and did not mix easily. The MLPA buffer is important in providing a suitable pH for MLPA reaction (PH around 8.2) to prevent depurination of DNA during the heat treatment at 98°C. The amount of polymerase enzyme during the PCR influences the relative probe signals, where most of the probes will have decrease relative signals with decrease polymerase activity. In both reactions, complete mixing of the dilution is crucial and could avoid a potential source of error.

The use of small volume of reagent and sample (ranging from 0.5 µl to 4.0 µl) in MLPA could also cause problems in accurate pipetting of the reagents. Excessive evaporation could occur during the 16-hour hybridization period of PCR, attributed by poor quality plastic or pressure from the heated lid, causing a much lower peak area of the longer fragment. For hybridization process, at least a volume of 5.5 µl should be retained after the overnight incubation at 60^{0}C for optimum results.[5] From our experience, preparation of a master mix instead of adding small volume of reagents consecutively into the tube, would ensure equal quantity of reagent dispersed and homogeneity between samples. Processing large amounts of specimen will also improve the accuracy of adding each component and thus ensuring reproducibility of the method.

MLPA analysis only requires as low as 20 ng of fetal DNA. Isolation of fetal DNA from amniotic fluid can be challenging especially with small amounts of fluid. In this study, the samples

from six cases had low DNA concentration. The MLPA quality control fragment (DQ fragment) in these samples indicated insufficient amount of genomic DNA. As a result, the probes did not hybridise to the targeted DNA sequence and no amplification products were generated. Although DNA is a stable molecule, DNA from amniotic fluid degrades easily. The amount of at least 5 ml of amniotic fluid and early processing is important to avoid PCR failures due to inadequate DNA.

From this study, all conclusive results by MLPA were comparable with the results from QF-PCR with 100% sensitivity and 100% specificity. There were no false negative or false positive results. For all three cases of trisomy 18, five of the eight chromosome 18 probes had shown relative probe signals equal to or more than 1.3 (ranging from 1.30 to 2.06). Only the NFATC1 probe was consistently positive in all three cases with the relative peak signal ranging from 1.32 to 1.50. Four probes showed positive result in two out of three cases, namely probe MC2R, SS18-1, SS18-2 and SERPINB2. The TYMS, SMAD4, PMAIP1 probes were only positive for one of the three cases. One case that was positive for trisomy 13 had shown positive signals in seven out of eight probes of chromosomes 13 and the relative peak signals ranged from 1.31 to 1.53. However, the only probe for chromosome 13 that was negative was DACH1 probe (relative peak of 1.21), and failure of certain probes to detect trisomies has been reported in other studies.[10] For chromosome 18 probes, none of the probe showed 100% sensitivity; SERPINB2 and DCC probes having the highest false negative results. The sensitivity of the probes ranged from 30% to 86%. For chromosome 13 probes, only 2 probes had shown 100% sensitivity (RB1 and ING1 probes). In another study,[11] it was also noted that not all probes were found to be abnormal in the trisomy cases. The findings that certain probes for trisomies detection showed a higher percentage of false negative results were unexpected because all probes were designed to detect a single copy sequence per haploid gene.[10] Variation of the probe performance may also be due to the polymorphism that occurs in targeted specific sequence and sub-optimization of the MLPA method.[11]

From analysis of samples with normal karyotype, all the probes for chromosome 21, 18 and 13 showed means of normalised peak ratio of approximately 1. There were some individual probe variations (standard deviation ranging from 0.07 - 0.3) and relative probe signals < 0.7 in normal samples may be due to incomplete denaturation of the target sequence area or due to a decrease hybridization efficacy of the probe to the target.[10] These findings might have also be due to chromosomal aberration such as point mutation which occurred in the nucleotide position in the target sequence that prevented hybridization of the probe.[12] Unexpected results could have also been due to large scale copy number variations of over 100kb of genomic DNA that have been shown to occur in normal individuals, and that could be found in all chromosomes.[13] An example is the Probe NFATC1, which is a specific probe for chromosome 18, is located within the region that is deleted or duplicated at a frequency of 1%.[10]

Both MLPA and QF-PCR have high throughput, where up to 96 samples can be tested simultaneously using 96 well thermocycler and capillary sequencer, which can also process smaller throughput for small laboratory that process small sample size. Commercial prepared kits are available for both methods and analysis can be conveniently performed by using semi-automated thermocycler for hybridization, ligation and amplification stages. The amplification product can be automatically separated by capillary electrophoresis and the final result can be interpreted by using computer software. With automation, the MLPA and QF-PCR are less laborious and the results are readily available within 24 to 48 hours compared to the conventional karyotyping.

MLPA have several advantages compared to QF-PCR. In MLPA, 40 oligonucleotide probes are used to detect 40 loci in a single reaction whereas QF-PCR is only limited to approximately 12 loci or less.[14] Moreover, increasing primers in multiplex PCR can cause problems due to primer-dimer interactions.[8] Furthermore, MLPA uses non polymorphic markers that is highly likely to be present in general population while the QF-PCR uses polymorphic short tandem repeat markers which show variable frequency in different populations and some of these markers can be non-informative if the patient is homozygous for that allele.[14]

In conclusion, the results of the study showed that MLPA is comparable with that of QF-PCR for the detection of common aneuploidies and would be another rapid and reliable tool for prenatal diagnosis.

REFERENCES

1. Jorde LB, Carey JC, Bamshad MJ, White RL. Medical genetic. 2nded. Philadelphia: Mosby; 2000.
2. Driscoll DA, Gross S. Clinical practice. Prenatal screening for aneuploidy. N Engl J Med. 2009; 360(24): 2556-62.
3. Snijders RJ, Noble P, Sebire N, Souka A, Nicolaides KH. UK multicentre project on assessment of risk of trisomy 21 by maternal age and fetal nuchal-translucency thickness at 10-14 weeks of gestation. Fetal Medicine Foundation First Trimester Screening Group. Lancet. 1998; 352(9125):343-6.
4. Wapner R, Thom E, Simpson JL, *et al*. First-trimester screening for trisomies 21 and 18. N Engl J Med.2003; 349(15): 1405-13.
5. Schouten J, Galjaard RJ. MLPA for prenatal diagnosis of commonly occurring aneuploidies. Methods Mol Biol. 2008; 444:111-22.
6. Van Opstal D, Boter M, de Jong D, *et al*. Rapid aneuploiy detection with multiplex ligation-dependent probe amplification:a prospective study of 4000 amniotic fluid samples. Eur J Hum Genet. 2009; 17(1):112-21.
7. Dudarewicz L, Holzgreve W, Jeziorowska A, Jakubowski L, Zimmermann B. Molecular methods for rapid detection of aneuploidy. J Appl Genet. 2005; 46(2):207-15.
8. Butler JM. Forensic DNA typing. 2nded. USA: Elsevier Academic Press; 2005.
9. Badenas C, Rodriguez-Revenga L, Morales C, *et al*. Assessment of QF-PCR as the first approach in prenatal diagnosis. J Mol Diagn. 2010; 12(6): 828-34.
10. Hochstenbach R, Meijer J, van den Brug J, *et al*. Rapid detection of chromosomal aneuploidies in uncultured amniocytes by multiplex ligation-dependent probe amplification (MLPA). Prenat Diagn. 2005; 25(11): 1032–9.
11. Slater HR, Bruno DL, Ren H, Pertile M, Schouten JP, Choo KH. Rapid, high throughput prenatal detection of aneuploidy using a novel quantitative method (MLPA). J Med Genet. 2003; 40(12):907-12.
12. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res. 2002; 30(12):e57.
13. Iafrate AJ, Feuk L, Rivera MN, *et al*. Detection of large-scale variation in the human genome. Nat Genet. 2004; 36(9): 949–51.
14. Cirigliano V, Voglino G, Canadas MP, *et al*. Rapid prenatal diagnosis of common chromosome aneuploidies by QF-PCR.Assessment on 18 000 consecutive clinical samples. Mol Hum Reprod. 2004; 10(11): 839–46.