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-
fication (MLPA) technique is a recent and sensitive molecular technique that involves relative quantification of 40 different nucleic acid sequences in one reaction. 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.

QF-PCR is another fast and specific method in detecting aneuploidies. 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 Qubit fluorometer. 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°C to denature the DNA, and cooled to 25°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°C followed by 6 hours at 60°C. After overnight incubation, heat stable ligase enzyme-65 was added for ligation process and the temperature was increased to 54°C for 15 minutes for optimal reaction, followed by ligase inactivation at 98°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°C for 1 minute, followed by 32 cycles amplification by PCR (30 seconds at 95°C, 30 seconds at 60°C and 60 seconds at 72°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°C followed by capillary analysis. For each run, two male controls were included.
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 $\leq 0.7$ and trisomy, by a relative probe signal $\geq 1.3$. The diagnosis of trisomy was made using the criteria that at least 4 out of 8 chromosome specific probes have signal $\geq 1.3$.

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 $\geq 1.3$ (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 coefficient variation result ranged from 1.31% to 12.8%.

Table 1: Relative probe signals in cases of trisomy 18

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

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

Table 2: Relative probe signals in cases of trisomy 13

<table>
<thead>
<tr>
<th>Chromosomes 13 probes</th>
<th>Relative probe signal</th>
<th>Case 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA2 probe</td>
<td>1.39</td>
<td></td>
</tr>
<tr>
<td>CCNA1 probe</td>
<td>1.38</td>
<td></td>
</tr>
<tr>
<td>RB1 probe</td>
<td>1.37</td>
<td></td>
</tr>
<tr>
<td>DLEU1 probe</td>
<td>1.31</td>
<td></td>
</tr>
<tr>
<td>DACH1 probe</td>
<td>1.21</td>
<td></td>
</tr>
<tr>
<td>ABCC4 probe</td>
<td>1.36</td>
<td></td>
</tr>
<tr>
<td>ING1 probe</td>
<td>1.53</td>
<td></td>
</tr>
<tr>
<td>ARHGEF7 probe</td>
<td>1.36</td>
<td></td>
</tr>
</tbody>
</table>

*Note: the shaded areas show relative probe signals of > 1.3
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°C for optimum results. 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

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

<table>
<thead>
<tr>
<th>MLPA probe</th>
<th>Chromosomal Position</th>
<th>PCR product size</th>
<th>Average probe signal</th>
<th>Standard deviation</th>
<th>Intra-assay precision (Coefficient variation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA2 probe</td>
<td>13q12.3</td>
<td>355</td>
<td>0.988</td>
<td>0.135</td>
<td>4.45%</td>
</tr>
<tr>
<td>CCNA1 probe</td>
<td>13q12.3</td>
<td>178</td>
<td>1.010</td>
<td>0.089</td>
<td>5.36%</td>
</tr>
<tr>
<td>RB1 probe</td>
<td>13q14.3</td>
<td>220</td>
<td>1.010</td>
<td>0.108</td>
<td>3.48%</td>
</tr>
<tr>
<td>DLEU1 probe</td>
<td>13q14.3</td>
<td>400</td>
<td>1.013</td>
<td>0.246</td>
<td>8.85%</td>
</tr>
<tr>
<td>DACH1 probe</td>
<td>13q21.3</td>
<td>265</td>
<td>0.993</td>
<td>0.077</td>
<td>5.83%</td>
</tr>
<tr>
<td>ABC4 probe</td>
<td>13q32</td>
<td>148</td>
<td>1.021</td>
<td>0.172</td>
<td>4.29%</td>
</tr>
<tr>
<td>ING1 probe</td>
<td>13q34</td>
<td>445</td>
<td>1.180</td>
<td>0.349</td>
<td>6.11%</td>
</tr>
<tr>
<td>ARHGEF7 probe</td>
<td>13q34</td>
<td>310</td>
<td>1.072</td>
<td>0.189</td>
<td>5.39%</td>
</tr>
<tr>
<td>TYMS probe</td>
<td>18p11.3</td>
<td>301</td>
<td>0.954</td>
<td>0.135</td>
<td>8.22%</td>
</tr>
<tr>
<td>MC2R probe</td>
<td>18p11.2</td>
<td>436</td>
<td>1.022</td>
<td>0.257</td>
<td>1.31%</td>
</tr>
<tr>
<td>SS18 - 1 probe</td>
<td>18q11.2</td>
<td>211</td>
<td>0.940</td>
<td>0.225</td>
<td>11.03%</td>
</tr>
<tr>
<td>SS18 - 2 probe</td>
<td>18q11.2</td>
<td>391</td>
<td>0.999</td>
<td>0.115</td>
<td>5.21%</td>
</tr>
<tr>
<td>SMAD4 probe</td>
<td>18q21.1</td>
<td>142</td>
<td>0.947</td>
<td>0.153</td>
<td>3.69%</td>
</tr>
<tr>
<td>PMAIP1 probe</td>
<td>18q21.1</td>
<td>172</td>
<td>0.966</td>
<td>0.112</td>
<td>4.32%</td>
</tr>
<tr>
<td>SERPINB2 probe</td>
<td>18q21.3</td>
<td>346</td>
<td>1.058</td>
<td>0.106</td>
<td>8.77%</td>
</tr>
<tr>
<td>NFATC1 probe</td>
<td>18q23</td>
<td>256</td>
<td>1.026</td>
<td>0.188</td>
<td>3.75%</td>
</tr>
<tr>
<td>STCH probe</td>
<td>21q11</td>
<td>247</td>
<td>0.939</td>
<td>0.115</td>
<td>4.54%</td>
</tr>
<tr>
<td>USP25 probe</td>
<td>21q11.2</td>
<td>202</td>
<td>0.909</td>
<td>0.142</td>
<td>3.70%</td>
</tr>
<tr>
<td>NCAM2 probe</td>
<td>21q21.1</td>
<td>166</td>
<td>0.955</td>
<td>0.147</td>
<td>2.36%</td>
</tr>
<tr>
<td>APP probe</td>
<td>21q21.3</td>
<td>337</td>
<td>1.104</td>
<td>0.099</td>
<td>7.58%</td>
</tr>
<tr>
<td>TIAM1 probe</td>
<td>21q22.1</td>
<td>427</td>
<td>1.084</td>
<td>0.186</td>
<td>7.24%</td>
</tr>
<tr>
<td>SOD1 probe</td>
<td>21q22.1</td>
<td>292</td>
<td>0.966</td>
<td>0.136</td>
<td>4.90%</td>
</tr>
<tr>
<td>SIM2 probe</td>
<td>21q22.2</td>
<td>136</td>
<td>1.010</td>
<td>0.358</td>
<td>12.84%</td>
</tr>
<tr>
<td>TFF1 probe</td>
<td>21q22.3</td>
<td>382</td>
<td>1.006</td>
<td>0.127</td>
<td>6.40%</td>
</tr>
</tbody>
</table>
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, 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