Performance of MLPA as a screening method for aneuploidy in uncultured amniocytes

Abstract

Objective: To test whether the Multiplex Ligation-dependent Probe Amplification (MLPA) technique can be used as a screening test for rapid diagnosis of aneuploidies in uncultured amniocytes.

Material and Methods: In this prospective blind study, MLPA with chromosomes 13, 18, 21, X and Y specific probe mixes was performed in 500 amniotic fluid samples. Chromosome copy numbers were determined by analyzing size and peak area for each MLPA probe. Results were compared with those of karyotyping/FISH.

Results: Conclusive test results were obtained in 98% of the samples, whereas 2% were inconclusive. In all conclusive tests, the MLPA results were concordant with that of cytogenetic and/or FISH analyses. There were no false-positive results. A case with 69,XXX triploidy resulted in the 1970s, and since then the most common indication for prenatal diagnosis remains an increased risk of having a child with trisomy syndromes of chromosomes 21, 13, 18 and sex chromosome aneuploidies. They account for 60-80% of abnormal fetal karyotypes detected in amniotic fluid cells (1). Fetal karyotyping has been the gold standard for diagnostic testing for over 30 years and no new technology could be proven usable for the detection of numerical and/or structural abnormalities for all chromosomes. However, a number of molecular methods based on uncultured fetal cells have been developed to reduce the waiting time related with prenatal chromosome analysis. Molecular methods including FISH and multiplex quantitative fluorescence-PCR (QF-PCR)

Introduction

Prenatal diagnosis for genetic disorders was first carried out in the 1970s, and since then the most common indication for prenatal diagnosis remains an increased risk of having a child with trisomy syndromes of chromosomes 21, 13, 18 and sex chromosome aneuploidies. They account for 60-80% of abnormal fetal karyotypes detected in amniotic fluid cells (1). Fetal karyotyping has been the gold standard for diagnostic testing for over 30 years and no new technology could be proven usable for the detection of numerical and/or structural abnormalities for all chromosomes. However, a number of molecular methods based on uncultured fetal cells have been developed to reduce the waiting time related with prenatal chromosome analysis. Molecular methods including FISH and multiplex quantitative fluorescence-PCR (QF-PCR) are now in common practice for the rapid prenatal diagnosis of the most prevalent chromosome abnormalities (2, 3). These technologies importantly decrease the reporting times from 2-3 weeks to 1-2 days (4-6). Although they are informative for only commonly seen aneuploidies, such a rapid result detection is commercially available. The experiences in prenatal samples using MLPA are promising, but further studies...
need to be reported to know the limitations and performance of the MLPA tests. In this prospective blind study, we present the results of 500 consecutive amniocentesis samples analyzed by computer assisted MLPA analysis. The sensitivity and specificity percentages of the technique and each of the probes were addressed in the study.

Materials and Methods

In this prospective blind study, a total of 500 amniotic samples were referred to the cytogenetics section of the Department for karyotyping. Referral reasons covered all the prenatal diagnosis indications including maternal age (≥35), increased Down Syndrome risk based on maternal serum screening and/or nuchal thickness measurement, ultrasound detected abnormalities or anxiety. The first 2 ml of amniotic fluid drawn was discarded because of maternal cell contamination. Usually, 15-20 ml of amniotic fluid samples were obtained and 2 ml was taken for MLPA testing. Blood contaminated samples were excluded. Of all samples, 450 (89%) were between 15 and 18 weeks whereas 80 were between 24 and 30 weeks. G-banding analysis and direct-FISH analysis by using AneuVision Probe Set (Vysis) were carried out by using standard techniques. Since the aim of this study was to test MLPA (SALSA MLPA kit P095 Aneuploidy Lot 0307, 1206, 1106, 0505) analysis prospectively, the MLPA data were interpreted without knowing FISH and/or karyotyping results.

Sample preparation and analysis

DNA from 2 μl amniotic fluid was isolated by using QIAamp kit (Qiagen) according to the manufacturer’s instructions. In total, 45-150 ng DNA was used in the MLPA protocol. The MLPA assay was performed according to the manufacturer’s protocol with small modifications. Briefly, 5 μl of lysate were denatured for 5 min at 98°C, and then 3 μl probe mix were added and the mix was heated at 95°C for 1 min and incubated at 60°C overnight (16 hours). By using mineral oil, the problems arising from evaporation were solved.

The ligation was performed at 540°C for 15 min by adding 32 μl heat-stable ligase-65 enzyme into the hybridization product. The reaction mix including the 10 μl ligation mix was preheated at 95°C for one minute, followed by 35 cycles (30 sec at 95°C, 30 sec at 60°C and 60 sec at 72°C). A measure of 2 μl PCR product was analyzed by capillary electrophoresis on an ABI Prism 310 Genetic Analyzer with Rox-500 size standards. DNA samples from three males and three females were spontaneously used as external normal controls.

MLPA data analysis

By visual analysis of peak profiles, test results were defined as conclusive if MLPA quality control fragments showing sufficient genomic DNA was present in the mixture. Genescan 3.7 and genotyper 3.6 software were used in the analysis of size and peak area for each MLPA probe and the data were exported to a Microsoft Excel based Coffalyser v1.4 program. For quantification purposes, the relative peak area for each probe was calculated as a fraction of the total sum of peak areas in a given sample. Each autosomal peak fraction was divided by the median peak fractions of that locus for all samples in that reaction. The relative probe signal values between 0.7 and 1.3 were defined as normal. If the value of target sequence was lower than 0.7, it was defined as monosomy, whereas trisomy was defined if the value is ≥1.3.

Results

The MLPA analyses were performed in 500 amniotic fluid samples. Conclusive test results were obtained in 98% of the samples, whereas 10 samples were “inconclusive”. The internal MLPA quality control fragments indicated an insufficient amount of genomic DNA in these 10 samples. However, no correlation was determined between the failure of MLPA analysis and gestational age at sampling. In all conclusive tests, the MLPA test for chromosomes 13, 18 and 21 was concordant with that of cytogenetic and/or FISH analyses. The criterion in the trisomy diagnosis was that at least four of eight chromosome-specific probes should have a relative probe signal higher than 1.3. By using this criterion, autosomal trisomy diagnosis was revealed in 24 samples (trisomy 21: 18 samples, trisomy 18: 4 samples and trisomy 13: 2 samples).

In the fetal sex determination, the presence of relative probe signals for X chromosome and Y chromosome specific probes were diagnosed as male, whereas the samples without Y chromosome specific signal but with X chromosome specific probe signals were diagnosed as female. All fetal sex results were consistent with the karyotyped sexes. In two cases, although there were no Y chromosome specific signals, relative probe signals specific to X chromosome were <1.3 in the range from 0.910 to 1.230. These two cases were diagnosed as monosomy X and the results were confirmed by the cytogenetic analysis. In one case, not only were there higher X chromosome specific signals in the ranges from 1.350 to 2.00, but also all Y chromosome specific signals (1.08-1.100) were seen and therefore the gonoosomal chromosome constitution of the case was diagnosed as XXX. The result was in accordance with the fetal karyotype (47, XXX). The diagnosis of the other case was XXX since the relative signal ratio of all eight X chromosome specific probes were ≥2.0 (ranges in between 2.00 and 3.170).

Although the sex chromosome constitution of the case was revealed as XX by the MLPA, the karyotype of the fetus was 69, XXX triploidy. The triploidy could not be diagnosed by the MLPA. Neither autosomal nor sex chromosomes specific probes showed higher relative probe signals. As seen in “Table 1”, the higher (≥1.3) relative probe signal ratio was only seen in the ABC4 probe specific to chromosome 13, but no higher ratio was determined in the other 23 autosomal specific probe signals. In the evaluation of the performance of the MLPA in aneuploidy screening, the sensitivity and specificity of the test were determined as 97% and 100%, respectively on the basis of 490 samples with conclusive results. As seen in “Table 2”, in a trisomy diagnosis of either chromosome 13, 18 or 21, almost all chromosome-specific probes have a relative probe signal >1.3. Besides, in the diagnosis of sex chromosome aneuploidies, all eight X chromosome-specific probes and four Y chromosome specific probes allowed a correct diagnosis “Table 3”. The sensitivity and false-positive rates for all autosomal trisomy probes were determined on the basis of the 490 samples with conclusive results, “Table 4”. While only 8 of 24 autosomal specific
probes have a sensitivity lower than 100%, the false-positive rate for all probes is below 0.1%. The sensitivity percentage of two chromosome 13 specific probes (85SPR and BRCA2) was 50% but their specificity was 100%. The highest false-positive rate was seen in ING1 chromosome 13 specific probe, the others were below 0.1%. The sensitivity rates of X chromosome-specific probes were 100%. However, the false-positive percentages of two X chromosome specific probes (AR and L1CAM) were higher compared to that of autosomal probes “Table 4”. Since it is a screening method for the detection of copy number alterations of chromosomes 13, 18, 21, X and Y, the structural aberrations of these chromosomes and aneuploidies other than these chromosomes could not be detected by this approach. In the clinical series of this study, a fetus with a balanced t(21;21) translocation and two fetuses with structural chromosome aberrations could be diagnosed by karyotyping.

Discussion

MLPA is a rapid technique for prenatal aneuploidy detection in a routine diagnostic laboratory. This is the first study in Turkey related to the data of MLPA used in a clinical series of 500 amniocentesis samples. Although the samples obtained from the 15th week to 30th week of gestation were analyzed, conclusive results were obtained in 98% of the samples and therefore the results showed that the MLPA test is usable until late pregnancy. Inconclusive results were obtained in 10 samples because of insufficient amount of DNA. However, no correlation was seen between the failure of MLPA tests and gestational age at sampling, but the cell content of the sample was an important factor for a reliable MLPA test. In six of these samples, the cell content of the fluid was significantly lower and there were some difficulties in karyotyping these samples, as well. The MLPA probe mix used in this study included four DQ (DNA Quantity) control fragments. These short fragments (in range 64-82 nt long) are very informative since they give off a clear warning signal if the amount of sample DNA is lower than the amount of DNA required for a reliable MLPA test (8). The amplification products of the DQ fragments are only visible when little or no DNA is present, and even when the ligation did not occur. Ten samples with inconclusive results in the present study showed the amplified DQ fragments. Our experiences showed that 2ml of amniotic fluid is sufficient to perform a conclusive test, but the cell content of the sample is becoming an important factor in the reliability of the test.

In the present study, the MLPA-diagnosed trisomic fetuses and fetuses with X chromosomal aneuploidies were confirmed by the cytogenetic and/or direct FISH analyses. However, a fetus with triploidy could not be diagnosed by MLPA but was determined by direct-FISH analysis in a 24h duration test. The inability to detecttriploidy is one of the main limitations of the MLPA assay and this has also been reported previously (9-13). If the sensitivity and specificity of the probes were evaluated individually, our experiences showed that most of the probes had 100% sensitivity. The lowest sensitivity rate was 50% seen in the P85SPR probe specific to chromosome 13 “Table 4”. The probes TYMS and SS18 specific to chromosome 18 had 75% sensitivity. However, the specificity of these probes was 100% on the basis of 490 samples with conclusive result. The false-positive rate of all probes was below 0.1%, but X chromosomal probes AR and L1CAM had higher false-positive rates than the other X-chromosome and autosomal specific probes “Table 4”. The high false-positive rate for X chromosomal specific probe

Table 4. Relative probe signals in a case with 69,XXX karyotype

| Probe Name | Chromosome | Length PCR | Ratio |
|------------|------------|------------|-------|
| SIM2       | 21q22.2    | 136        | 0.840 |
| MADC4      | 18q21.1    | 142        | 0.910 |
| ABCC4      | 13q32      | 148        | 1.390 |
| AR         | Xq11.2     | 154        | 1.480 |
| SRY        | Yp11.3     | 160        | 0.000 |
| NCAM2      | 21q21.1    | 166        | 1.14  |
| PMAIP1     | 18q21      | 172        | 1.05  |
| CCNA1      | 13q12.3    | 178        | 0.700 |
| FAACL4     | Xq23       | 184        | 1.660 |
| SRY        | Yp11.3     | 193        | 0.000 |
| USP25      | 21q11.2    | 202        | 1.080 |
| SS18       | 18q11.2    | 211        | 0.730 |
| RB1        | 13q14.3    | 220        | 0.890 |
| ARX        | Xp22.1     | 229        | 1.410 |
| UTY        | Yq11       | 238        | 0.000 |
| STCH       | 21q11      | 247        | 0.870 |
| NFATC1     | 18q23      | 256        | 0.940 |
| DACH       | 13q21.3    | 265        | 1.06  |
| TM4SF2     | Xp11.4     | 274        | 1.550 |
| ZFY        | Yp11.3     | 283        | 0.00  |
| SOD1       | 21q22.1    | 292        | 0.950 |
| TYMS       | 18p11.3    | 310        | 0.890 |
| P5SSPR     | 13q34      | 310        | 1.010 |
| L1CAM      | Xq28       | 319        | 1.600 |
| APP        | 21q21.3    | 337        | 1.260 |
| SERPINB2   | 18q21.3    | 346        | 1.12  |
| BRCA2      | 13q12.3    | 355        | 1.16  |
| RPS6KA3    | Xp22.2     | 364        | 1.210 |
| TFF1       | 21q22.3    | 382        | 1.110 |
| SS18       | 18q11.2    | 391        | 0.780 |
| DLEU1      | 13q14.3    | 400        | 0.82  |
| PDCD8      | Xq25       | 409        | 1.600 |
| TIA1       | 21q22.1    | 427        | 1.000 |
| MC2R       | 18p11.2    | 436        | 0.95  |
| ING1       | 13q34      | 445        | 1.210 |
| DMD        | Xp21.2     | 454        | 1.530 |
AR has also been reported previously (10). These unexpected false-positive data might be due to recently detected large-scale copy-number variations (LCV) or copy-number polymorphisms (CNP) spanning from several kilobases to megabase pairs of DNA (14-17). However, population-specific variations might also be involved in these false-positive results. Mutations or polymorphisms very close to the probe ligation site may cause a reduced peak area. In MLPA, amplification of probes by PCR

depends on the presence of small specific target sequences in the sample. Nucleotide mismatches at the probe binding site prevent probe hybridization and ligation and therefore single base changes may result in deletions (7, 18). Therefore, in these variations, the relative signal ratios of the other probes specific to the related chromosome should be analyzed in detail.

The widely diverging sensitivity of the MLPA probes in aneuploidy screening has been discussed in previous studies (7, 10, 19). Slater et al. (19) reported false-negative results but they did not document the probes, whereas the false-positive rate in the study of Hochstenbach et al. (10) was between 0.0% and 4.2% and they reported that only a few probes have 100% sensitivity. The differences might also be due to the differences in probe mixtures, since the SALSA P095 probe mix used in this study is an improved version of the old SALSA MLPA P001 probe mix and it has been mentioned in the MRC-Holland page (8) that the new version is less sensitive to variations in the quality of DNA.

In conclusion, the high sensitivity and specificity rates and low failure rate showed that the MLPA assay can be used as a rapid aneuploidy screening test in uncultured amniocytes. The test is inexpensive and the result can be revealed in 2-3 days, which is very helpful for parental anxiety. However, the inability of the test to detect structural chromosome abnormalities, chromosome aneuploidies other than common chromosome syndromes and the mosaic status of fetus must always be taken into consideration. Because of these limitations, we suggested that the MLPA assay can be performed in clinical diagnostic laboratories together with fetal karyotyping.

**Conflict of interest**

No conflict of interest is declared by authors.

Table 2. Relative probe signals in fetuses with trisomy 13, 18 and 21

| Tri-13 Probes | 1234 | 1350 | Tri-18 probes | 1506 | 1631 | 1041 | 1640 |
|---------------|------|------|---------------|------|------|------|------|
| ABC24         | 1.620| 1.360| MADH4         | 1.670| 1.790| 1.370| 1.600|
| CCNA1         | 1.640| 1.720| PMAIP1        | 1.50 | 1.53 | 1.47 | 1.38 |
| RB1           | 1.640| 1.480| SS18          | 1.40 | 1.35 | 1.410| 1.360|
| DACH          | 1.36 | 1.39 | NFATC1        | 1.310| 1.350| 1.410| 1.480|
| P 85SPR       | 1.460| 1.290| TYMS          | 1.05 | 1.360| 1.460| 1.360|
| BRCA2         | 1.48 | 1.284| SERPINB2      | 1.32 | 1.43 | 1.36 | 1.40 |
| DLEU1         | 1.40 | 1.39 | SS18          | 1.40 | 1.190| 1.310| 1.600|
| ING1          | 1.780| 1.640| MCZR          | 1.36 | 1.31 | 1.43 | 1.38 |

| Tri-21 Probes | 803 | 811 | 1128 | 1415 | 1868 | 1591 | 1770 | 1864 | 1499 | 1948 | 1954 | 1014 | 1316 | 1358 | 1367 | 1527 | 1670 | 1888 |
|---------------|-----|-----|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| SIM2          | 1.57| 1.420| 1.380| 1.800| 1.400| 1.400| 1.350| 1.400| 1.340| 1.380| 1.40 | 1.50 | 1.42 | 1.56 | 1.50 | 1.40 | 1.36 |
| NCAM2         | 1.74| 1.47 | 1.37 | 1.70 | 1.92 | 1.52 | 1.40 | 1.42 | 1.92 | 1.37 | 1.82 | 1.64 | 1.33 | 1.59 | 1.54 | 1.39 | 1.46 | 1.32 |
| 4SP25         | 1.590| 1.460| 1.360| 1.60 | 1.60 | 1.360| 1.460| 1.850| 1.600| 1.360| 1.480| 1.430| 1.310| 1.390| 1.340| 1.480| 1.340| 1.310 |
| STCH          | 1.600| 1.290| 1.400| 1.830| 1.32 | 1.580| 1.390| 1.420| 1.370| 1.090| 1.370| 1.310| 1.360| 1.420| 1.480| 1.210| 1.340| 1.320 |
| SOO1          | 1.610| 1.320| 1.420| 1.420| 1.35 | 1.500| 1.50 | 1.200| 1.560| 1.520| 1.560| 1.830| 1.340| 1.400| 1.340| 1.440| 1.340| 1.460 |
| APP           | 1.700| 1.430| 1.330| 1.530| 1.430| 1.350| 1.530| 1.42 | 1.340| 1.300| 1.340| 1.620| 1.340| 1.600| 1.260| 1.360| 1.400| 1.380 |
| TFF1          | 1.870| 1.320| 1.520| 1.420| 1.510| 1.390| 1.450| 1.38 | 1.800| 1.420| 1.800| 1.410| 1.480| 1.450| 1.380| 1.40 | 1.480| 1.380 |
| TIAM1         | 1.450| 1.500| 1.450| 1.450| 1.330| 1.500| 1.530| 1.42 | 1.380| 1.420| 1.380| 1.390| 1.190| 1.450| 1.520| 1.500| 1.420 |

Table 3. Relative probe signals in fetuses with sex chromosomal aneuploidies

| Probes | Chromosome | Monosomy X | XXY | XXX |
|--------|------------|------------|-----|-----|
| AR     | 1788       | 1953       | 1778| 1003|
| SRY    | 0.980      | 1.770      | 2.140|
| FACL4  | 1.080      | 1.490      | 3.170|
| SRY    | 1.000      | 1.100      | 0.000|
| ARX    | 1.010      | 1.430      | 3.050|
| UTY    | 1.070      | 0.000      | 0.000|
| TM4SF2 | 1.230      | 2.000      | 2.570|
| ZFY    | 1.090      | 1.08       | 0.000|
| L1CAM  | 1.000      | 1.760      | 2.000|
| RPS5K3 | 1.040      | 1.350      | 2.890|
| PDCD8  | 0.930      | 1.970      | 2.840|
| DMD    | 0.910      | 1.350      | 2.040|

AR has also been reported previously (10). These unexpected false-positive data might be due to recently detected large-scale copy-number variations (LCV) or copy-number polymorphisms (CNP) spanning from several kilobases to megabase pairs of DNA (14-17). However, population-specific variations might also be involved in these false-positive results. Mutations or polymorphisms very close to the probe ligation site may cause a reduced peak area. In MLPA, amplification of probes by PCR
Table 4. Performance of probes in detection of autosomal and sex chromosomal aneuploidies

| Tri-13 Probes | Sensitivity | False-positive | Tri-18 probes | Sensitivity | False-positive | Tri-21 probes | Sensitivity | False-positive |
|---------------|-------------|----------------|---------------|-------------|----------------|---------------|-------------|----------------|
| ABCC4         | 100         | 0.00           | MADH4         | 100         | 0.04           | SIM2          | 100         | 0.01           |
| CCNA1         | 100         | 0.07           | PMAIP1        | 100         | 0.00           | NCAM2         | 100         | 0.00           |
| RB1           | 100         | 0.00           | SS18          | 100         | 0.08           | 4SP25         | 100         | 0.02           |
| DACH          | 100         | 0.1            | NFATC1        | 100         | 0.02           | STCH          | 85.7        | 0.06           |
| P85SPR        | 50          | 0.00           | TMYs          | 75          | 0.09           | SOO1          | 95.2        | 0.00           |
| BRCA2         | 50          | 0.00           | SERPINB2      | 100         | 0.00           | APP           | 95.2        | 0.08           |
| DLEU1         | 100         | 0.00           | SS18          | 75          | 0.00           | TFF1          | 100         | 0.00           |
| ING1          | 100         | 0.14           | MC2R          | 100         | 0.00           | TIAM1         | 95.2        | 0.09           |

| X chromosome Specific Probes | 45,X Sensitivity | False-positive | XXY Sensitivity | False-positive | XXX Sensitivity | False-positive |
|------------------------------|------------------|----------------|-----------------|----------------|-----------------|----------------|
| AR                           | 100              | 0.00           | 100             | 0.02           | 100             | 0.5            |
| FACLI4                       | 100              | 0.08           | 100             | 0.00           | 100             | 0.00           |
| ARX                          | 100              | 0.03           | 100             | 0.00           | 100             | 0.02           |
| TM4SF2                      | 100              | 0.00           | 100             | 0.00           | 100             | 0.03           |
| L1CAM                       | 100              | 0.00           | 100             | 0.00           | 100             | 0.4            |
| RPS6KA3                     | 100              | 0.02           | 100             | 0.00           | 100             | 0.00           |
| PDCD8                       | 100              | 0.00           | 100             | 0.00           | 100             | 0.00           |
| DMD                         | 100              | 0.00           | 100             | 0.09           | 100             | 0.02           |

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