Določanje anevploidij z metodo pomnoževanja od ligacije odvisnih prob v fetalnih tkivih splavkov

Detection of aneuploidy using multiplex ligation–dependent probe amplification in fetal tissues from aborted pregnancies

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Izvleček

Namen: *Spontani splavi se pojavljajo v približno 10–15% prepoznavnih nosečnosti. V prvem trimesečju je približno ≈50% splavov posledica kromosomskih napak, v večini primerov so to kromosomske anevploidije. Klasična metoda določanja anevploidij je citogenetska analiza. Citogenetska analiza zgodnjih spontanih splavov je težavna zaradi pogoste odsotnosti celične rasti ali slabe kvalitete kromosomov. V teh primerih se poslužujemo drugih metod, neodvisnih od rasti celične kulture. V študiji smo uporabili metodo pomnoževanja od ligacije odvisnih prob (MLPA). V primerjavi s klasično citogenetsko analizo smo na vzorcu embrionalnih tkiv potrdili ustreznost in kompatibilnost metode.*

Metode: *Vsi vzorci embrionalnih tkiv po spontanih splavih so bili kultivirani, kariotipizirani, prav tako je bila izolirana genomska DNA. Za MLPA*

Abstract

Purpose: *About 10–15% of all pregnancies terminate as spontaneous miscarriages. In the first trimester, ≈50% of spontaneous miscarriages are the result of chromosomal aberrations, mostly chromosomal aneuploidies. Cytogenetic analyses are used to confirm aneuploidy in failed pregnancies. Culture failure or poor–quality chromosomes are often problems in those cases. In such situations, methods that are independent of tissue culture are used, and we employed multiplex ligation–dependent probe amplification (MLPA). We determined if MLPA is an appropriate and compatible method compared with classical cytogenetic analyses on fetal tissues.*

Methods: *All fetal samples received from spontaneous abortions were cultured, karyotyped (if possible) and genomic DNA extracted. MLPA*

analizo smo uporabili komercialne komplete s subtelomerno specifičnimi DNA sondami. V primeru odsotnosti celične rasti so bile anevploidije ugotovljene z MLPA analizo, potrjene s primerjalno genomsko hibridizacijo (PGH).

Rezultati: *MLPA analiza je potrdila neuravnotežene kromosomske nepravilnosti, ugotovljene s citogenetsko analizo pri vseh vzorcih, kjer je bila uspešna celična rast, in hkrati omogočila analizo v primerih, kjer celična rast ni bila uspešna. Ugotovljene so bile mnoge številčne kromosomske spremembe, redke trisomije in druge neuravnotežene kromosomske preureditve.*

Zaključek: *MLPA analiza omogoča pridobitev informacij o številu kromosomov v primerih, ko citogenetska analiza ni možna zaradi odsotnosti celične rasti ali slabe kvalitete kromosomov. Iz dobljenih rezultatov ugotavljamo, da je MLPA potencialno tudi zelo uporabna metoda za hitro in kvalitetno prenatalno diagnostiko.*

analyses were undertaken using subtelomeric probe kits. Additionally, comparative genomic hybridization (CGH) was used to confirm aneuploidy detected by MLPA in cases of failed culture growth.

Results: *MLPA analyses confirmed an unbalanced chromosome abnormality identified by cytogenetic analyses in all cases in which tissue culture was successful, and provided data in cases of failed culture growth. Several common numeric chromosome aberrations were detected, as well as rare trisomies and other unbalanced chromosome rearrangements.*

Conclusions: *MLPA analyses can provide information about the karyotype of a DNA sample if cytogenetic analyses are not possible because of a lack of viable cells or if only a small amount of genomic DNA is available. These data indicate that MLPA may also be a very useful method for early prenatal aneuploidy screening.*

INTRODUCTION

Chromosome aberrations are the most important cause of abnormal development in early human life. Aneuploidy is present in ≈60% of spontaneous abortions, which occur predominantly in the first trimester (1). Most cases (>85%) are due to numeric abnormalities (which include autosomal trisomies and 45,X, monosomy) as well as polyploidy, whereas structural chromosomal changes can be observed in ≈5% (2, 3). Chromosome aberrations are also frequently detected in pregnancies with diagnosed congenital anomalies, and are an important reason for elective late terminations of pregnancy (2, 4).

After >30 years of successful application, cytogenetic analyses remain the method of choice for the identification of aneuploidy. Despite being time–consuming, labor–intensive and tissue culture–dependent, karyotyping is the "gold" standard and all other methods can be used only as rapid screening methods preceding it (5). Different approaches have been developed to detect aneuploidy faster and without employing tissue culture (6). Quantitative–fluorescent polymerase chain reaction (QF–PCR) (7) and interphase fluorescent in–situ hybridization (interphase FISH) (8) are the most frequently used methods for rapid detection of trisomies of chromosomes 13, 18 and 21, as well as numerical aberrations of sex chromosomes. Both methods have been extensively tested and have a place in routine prenatal genetic laboratory analyses (9–13). QF–PCR and FISH are usually employed to detect the most common trisomies, but each method can also identify other numeric chromosome aberrations (14, 15). Among available methods, comparative genomic hybridization can be used to detect chromosome aberrations (16, 17), whereas real–time PCR (18, 19) has been adopted for identification of aneuploidy. Increasingly popular array–based methods such as array– comparative genomic hybridization (CGH) analyses can also provide a wealth of information on the number and structure of chromosomes (20, 21, 22). The spectrum of methods that can be used to identify aneuploidy has been recently augmented by multiplex ligation–dependent probe amplification (MLPA) (23). MLPA was developed for accurate detection of copy number variations in a sample of extracted nucleic ac-

ids. Up to 50 loci can be simultaneously analyzed in a single MLPA reaction, and quantitative differences in the genetic material present in prenatal aneuploidy cases can be readily and reliably detected. MLPA has been successfully used for the screening of the most common aneuploidies (trisomies of chromosomes 13, 18, and 21) (24, 25) in amniotic fluid samples. For this analysis, kits containing chromosome–specific probes (P001, P095) are available from the manufacturer: MRC–Holland (Amsterdam, the Netherlands; www.mrc–holland.com). However, a single MLPA reaction enables the quantification of all chromosomes if kits with subtelomeric probes (P019/P020, P036B, P069, P070; MRC–Holland) are used for the analysis. These kits were developed for the analysis of subtelomeric regions of chromosomes that are prone to variation in copy number (26). The kits (which contain probes from all subtelomeric regions) have been used in studies of idiopathic mental retardation in humans (27, 28). In the case of an altered number of a particular chromosome, MLPA analyses with a subtelomeric kit would show a change in the quantity of both specific probes, which are located on different arms of the chromosome. In addition, any unbalanced structural chromosome rearrangements that involve subtelomeric regions would be observed. Therefore, a single MLPA reaction should permit the detection of ≈80% (2, 5) of prenatal chromosome aberrations.

In the present study, the ability of MLPA to identify aneuploidy was compared with cytogenetic analyses on fetal tissues from terminated pregnancies.

MATERIAL AND METHODS

Ethical approval of the study protocol

Ethical approval was granted from University Clinical Centre Maribor (Maribor, Slovenia). All patients provided written informed consent to be involved in the study.

Samples

A total of 71 cases of spontaneously aborted pregnancies or induced abortions because of diagnosed severe malformations were included. Tissue samples were chorionic villi or fetal skin. These samples are routinely sent for karyotyping in the Medical Genetics Laboratory, Maribor Teaching Hospital (Maribor, Slovenia). Samples of peripheral venous blood were obtained from parents for karyotyping in several cases to clarify the results of fetal tissue analyses.

Cytogenetic analyses

Fetal tissues and blood were cultured and chromosomes harvested according to standard cytogenetic procedures. They were analyzed by the GTG banding method (G bands produced with Giemsa and trypsin).

DNA extraction

Genomic DNA was extracted from tissue samples using a modified method (29). Briefly, ≤100 mg of fetal tissue was incubated overnight in lysis buffer (5% sodium dodecyl sulfate (SDS), 10 mM NaCl, 10 mM tris–HCl, 166.7 µg/ml proteinase K) at 37°C. DNA was then precipitated from the supernatant after the addition of 9.5 M ammonium acetate and ice–cold 2–propanol. It was then dried and dissolved in Tris–ethylenediamine tetra–acetic acid (EDTA) storage buffer (10 mM Tris–HCl, 1 mM EDTA).

MLPA

MLPA was undertaken using commercial MLPA kits containing subtelomeric probes (P0036B, P070; MRC Holland) according to manufacturer protocols (23). Briefly, probe hybridization on sample DNA was carried out overnight, followed by ligation of annealed probes using a thermostable ligase enzyme. The ligation products were amplified by PCR with one D4–labeled and one unlabeled oligonucleotide. The analysis of PCR products was done using a Beckman–Coulter CEQ8000 capillary electrophoresis system (Beckman–Coulter, Brea, CA, USA). All samples were tested with both kits, and samples with an abnormal result were retested to confirm the MLPA analysis.

Analyses of MLPA data

Data from capillary electrophoresis were processed with CEQ8000 software for fragment analyses. These data were then exported to a Microsoft Excel spreadsheet program to complete the analyses. To produce normalized ratios reflecting the relative probe dosage,

each peak area was divided by the sum of all peaks from the sample trace. For each probe, this ratio was divided by the same ratio from an unaffected control DNA run in the same experiment. An expected normalized value of 1.0, a ratio <0.8 in the case of a deletion (monosomy) and a ratio >1.3 for a duplication (trisomy) were adopted for the study.

CGH

Samples with abnormal MLPA results but unavailable karyotypes were also analyzed by CGH for additional confirmation. CGH was done as described previously (30). Briefly, the patient's DNA and normal reference DNA were labeled with Spectrum Green and Spectrum Orange (Vysis, Chicago, IL, USA). A total of 1 µg of DNA and 30 µg Cot1 DNA were hybridized to normal metaphase chromosomes. Slides were hybridized for 3 days, washed and counterstained with 4,6–diamidino–2–phenylindole (DAPI). CGH image capture was undertaken with the Cytovision system (Applied Imaging, San Jose, CA, USA) interfaced with a fluorescence microscope (Axioplan; Zeiss, Oberkochen, Germany). In each case, ≥10 metaphases were analyzed. The average green–to–red ratio fluorescence intensity ratio profile was calculated for each chromosome. In regions of normal sequence copy numbers, the average green–to–red ratio was found to be ≈1.0. Chromosomal regions with a ratio >1.2 were considered to be gained, whereas regions with a ratio below 0.8 were deemed to be deleted (31).

RESULTS

A total of 71 samples of fetal tissue (Table 1), 34 samples of chorionic villi and 37 samples of fetal skin received from the Gynecology and Perinatology Department of Maribor Teaching Hospital were included in the study. In 10 cases, pregnancy was achieved using IVF procedures. The mean duration of gestation was 14.71 weeks (range, 7–34 weeks). The pregnancy was artificially terminated in 24 cases, whereas it ended spontaneously in 47 cases. The mean age of women was 32.24 years (range, 23–45 years).

Cytogenetic analyses were undertaken in 53 cases, whereas in 18 cases tissue culture was unsuccessful. MLPA analyses were done on all 71 samples. Both

Table 1. *Clinical data on included samples*

Number of cases	71
Chorionic villi	34
Fetal skin	37
IVF procedures	10
Duration of gestation (weeks)	14.7 (range, 7–34)
Spontaneous abortion	47
Artificial termination of pregnancy	24
Average maternal age (years)	32.2 years (range, 23–45)

methods were used to identify: 19 normal female karyotypes (46,XX); 10 normal male karyotypes (46,XY); 4 trisomies of chromosome 21; 2 trisomies of chromosome 18, 2 monosomies of chromosome X; 1 double trisomy of chromosomes 20 and 21 (Figure 1); 1 trisomy of chromosome 16; 1 trisomy of chro-

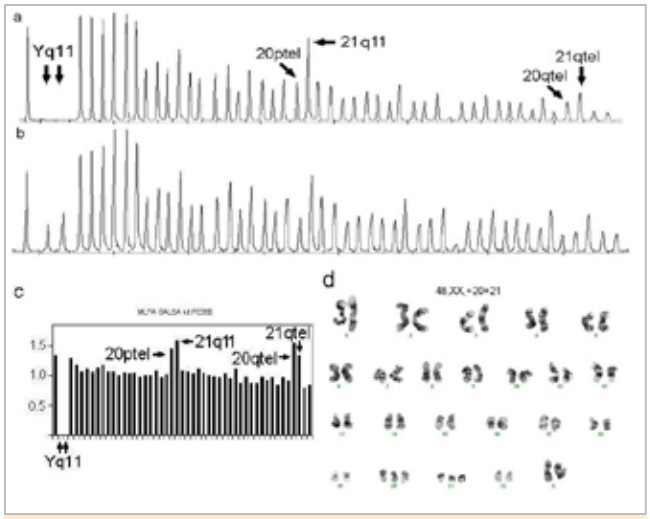

Figure 1. *Trisomies of chromosome 20 and 21 in samples of female fetal tissue. (a) Case electrophoretogram: arrows point to absent signals from Yq11–specific probes and to increased signals from 20ptel–, 20qtel–, 21q11– and 21qtel–specific probes compared with control signals (kit P036B; MRC–Holland). (b) Control electrophoretogram. (c) Graphic presentation of ratios between the case and the control. (d) Karyotype 48,XX,+20,+21.*

mosome 15; 1 trisomy of chromosome 12; 1 trisomy of chromosome 2; 1 case of 47,XXX; and 1 case of 47,XXY. Both unbalanced chromosome rearrangements (45,XY,der(14),t(14;18)(q10;q10), 46,XX,del(4)(q32.2),dup(16)(qtel)) (Figure 2) were correctly detected by MLPA as quantitative changes in the subtelomeric regions of the rearranged chromosomes. Additional cytogenetic analyses of the parents showed that the rearrangement between chromosomes 14 and 18 was *de novo*, whereas the rearrangement between chromosomes 4 and 16 was inherited from the father. MLPA analyses identified a possible trisomy of chromosome 21, but the existence of isochromosome i(21q) was not observed. Karyotyping detected 1 case with 46,XY,inv(2) and 2 cases of pericentric inversion on chromosome 9 (46,XX,inv(9)(p11;q13); 46,XY,inv(9)(p11;q13)), which could not be detected by MLPA analyses. In 1 case of XX/XY mosaicism observed on cytogenetic analyses, MLPA analyses yielded a result compatible with a normal male karyotype (signals for chromosome Y–specific probes were ≥1.0). In addition, a normal female karyotype was found on MLPA analyses to correspond to a normal male (chromosome Y–specific signals were not decreased; data not shown).

In 18 cases with failed tissue culture in which a cytogenetic result could not be obtained, MLPA analyses identified 10 normal females, 4 normal males and 4 chromosomal abnormalities: 1 trisomy of chromosome 16, 1 trisomy of chromosome 13, 1 trisomy of chromosome 10 (Figure 3) and 1 duplication of the 3qtel region (Figure 4). CGH analyses confirmed the presence of excess chromosomal material in these four cases (Figures 3 and 4). Duplication of the 3q subtelomeric region corresponded to the possible karyotype 46,XY,der(15),t(3,15)(q26.2;p11.2), which occurred because the mother was a carrier of the 46,XX,t(3;15)(q26.2;p11.2) chromosome rearrangement.

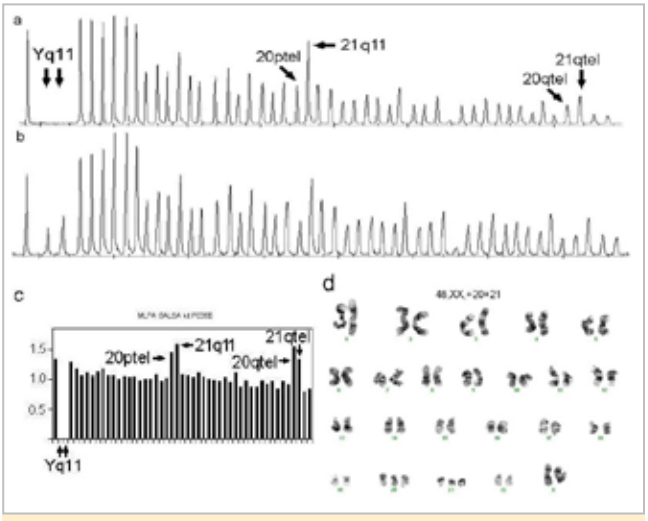

Figure 2. *Partial monosomy of 4q and partial trisomy of 16q in samples of female fetal tissue. (a) Case electrophoretogram: arrows point to absent signals from Yq11–specific probes, to a decreased signal from the 4qtel–specific probe, and to an increased signal from the 16qtel–specific probe when compared with control signals (kit P070; MRC–Holland). (b) Control electrophoretogram. (c) Graphic presentation of ratios between the case and the control; d) karyotype 46,XX,del(4)(q32.2),dup(16)(qtel) (father's karyotype was 46,XY,t(4;16)(q32.2;q24)).*

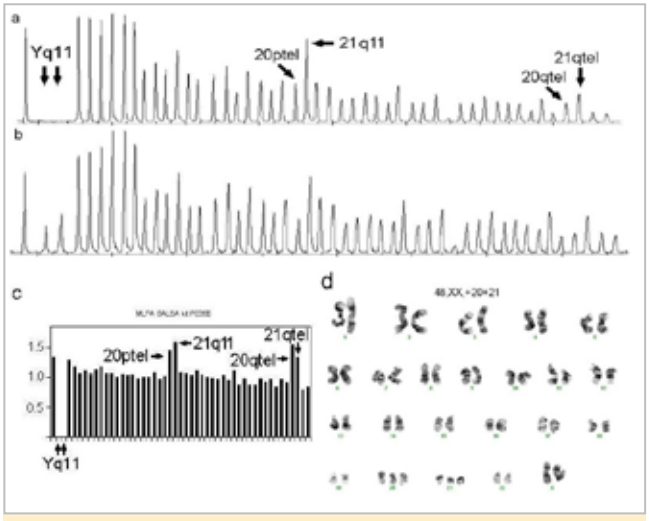

Figure 3. *Trisomy of chromosome 10 in samples of male fetal tissue (possible karyotype 47,XY,+10). (a) Case electrophoretogram: arrows point to signals from Yq11–specific probes and increased signals from 10qtel– and 10ptel–specific probes when compared with control signals (kit P070; MRC–Holland). (b) Control electrophoretogram. (c) Graphic presentation of ratios between the case and control. (d) CGH densitograms: gain of whole chromosome 10, presence of whole chromosome Y and loss of whole chromosome X (control DNA was normal female; CGH analyses were done on male chromosome spreads).*

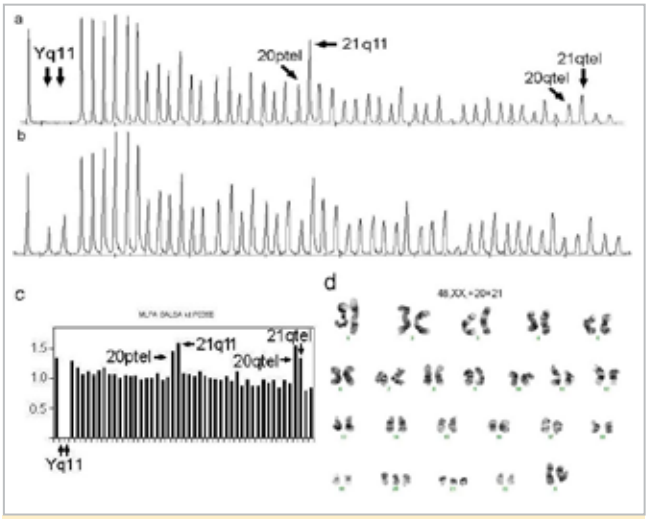

Figure 4. *Partial trisomy of 3q in samples of male fetal tissue (possible karyotype 46,XY,der(15),t(3,15)(q26.2;p11.2); mother's karyotype 46,XX,t(3;15)(26.2;p11.2)). (a) Case electrophoretogram: arrows point to signals from Yq11–specific probes and an increased signal from the 3qtel–specific probe when compared with control signals (kit P070; MRC–Holland). (b) Control electrophoretogram. (c) Graphic presentation of ratios between the case and control. (d) CGH densitograms: gain of 3q, presence of whole chromosome Y and loss of whole chromosome X (control DNA was normal female; CGH analyses were done on male chromosome spreads).*

DISCUSSION

We evaluated the ability of MLPA to detect unbalanced chromosome rearrangements in fetal tissue samples. The MLPA method was compared with the gold–standard method: cytogenetic analyses. All quantitative changes of chromosomal material found by cytogenetic analyses were successfully identified by MLPA analyses (Table 2). Although MLPA has been used to detect aneuploidy in fetal samples (amniotic fluid) (24, 25), our results showed that a single MLPA reaction could be used to identify all unbalanced numerical chromosome abnormalities in a test sample of DNA. Because this type of genetic change is by far the most important in spontaneous abortions, MLPA may be used as an alternative to karyotyping of fetal tissues from terminated pregnancies.

MLPA analyses also successfully identified 3 cases of unbalanced structural chromosomal rearrangements (Table 2). The identification succeeded because subtelomeric regions were involved and the MLPA kits used included probes from these regions. Some unbalanced rearrangements can be identified by MLPA as a change in a single signal (peak) (Figure 4). These results have to be differentiated from false–positives which can be observed in MLPA analyses or from common polymorphisms typical of subtelomeric regions (32). To clarify such findings, MLPA kits with different probes can be used or another method (i.e., CGH) can be applied. If both available MLPA subtelomeric kits (P036B, P070) are used and they provide concordant abnormal results for a single chromosome arm, this indicates the presence of a copy number change of that particular subtelomeric region. The physical size of the affected DNA fragment may be >50 kbp (see product manuals for probe locations at www.mrc–holland.com). Such results in fetal tissue from a failed pregnancy warrant further investigation of the parents, including karyotyping. In 2 of the 3 cases, one of the parents was the carrier of a balanced reciprocal translocation and pregnancy failure was the consequence of inheritance of an unbalanced set of chromosomes (Table 2).

Although subtelomeric regions are involved in most cases of unbalanced translocations, other rearrangements are also possible. MLPA analyses, as used in the present study, cannot be used to detect chromosome changes that are present elsewhere along the chromosomes. With cytogenetic analyses, 2 cases of pericentric inversion on chromosome 9 and 1 pericentric inversion on chromosome 2 were detected. More importantly, MLPA analyses also failed to detect the presence of an isochromosome, i(21q), although the additional copy of the long arm of chromosome 21 was correctly identified (Table 2). In the case with a karyotype 46,XX,i(21q), further investigation of the parents is warranted. One of them may be a carrier of the isochromosome and the necessary karyotyping may be omitted if only the MLPA result is available. Another important chromosome abnormality, polyploidy, would also be missed by MLPA analyses (2, 5). If fetal cells contain at least one additional complete set of chromosomes, then MLPA analyses would not de-

Table 2. *Results of karyotyping and MLPA analyses*

Karyotype	MLPA	MLPA Yq11	Cases
46,XX	No change	—	19
46,XY	No change	+	10
46,XX	No change	+	1
46,XX/46,XY	No change	+	1
46,XX, inv(9)(p11q13)	No change	—	1
46,XY, inv(9)(p11q13)	No change	+	1
48,XX,+20,+21	Gain 20ptel, gain 20qtel, Gain 21q11, gain 21qtel	—	1
47,XX,+21	Gain 21q11, gain 21qtel	—	2
47,XY,+21	Gain 21q11, gain 21qtel	+	2
46,XX,i(21q) a	Gain 21q11, gain 21qtel	—	1
47,XX,+18	Gain 18ptel, gain 18qtel	—	2
47,XY,+16	Gain 16ptel, gain 16qtel	+	1
47,XX,+15	Gain 15q11, gain 15qtel	—	1
47,XY,+13	Gain 13q11, gain 13qtel	+	1
47,XX,+12	Gain 12ptel, gain 12qtel	—	1
47,XX,+2	Gain 2ptel, gain 2qtel	—	1
47,XXY	Gain Xptel/Yptel, Gain Xqtel/Yqtel	+	1
47,XXX	Gain Xptel/Yptel, Gain Xqtel/Yqtel	—	1
45,X	Loss Xptel/Yptel, Loss Xqtel/Yqtel	—	2
46,XY,inv(2) a	No change	+	1
45,XY,der(14), t(14;18)(q10;q10) b	Loss 18ptel	+	1
46,XX, del(4)(q32.2),dup(16)(qtel) c	Loss 4qtel, gain 16qtel	+	1
No growth	Gain 16ptel, gain 16qtel d	+	1
No growth	Gain 13q11, gain 13qtel d	+	1
No growth	Gain 10ptel, gain 10qtel d	+	1
No growth	Gain 3qtel d, e	+	1
No growth	No change	—	10
No growth	No change	+	4
		Total	71

[a] *No data available from parents*
[b] *de novo rearrangement*
[c] *Father's karyotype was 46,XY,t(4;16)(q32.2;q24)*
[d] *Confirmed using CGH*
[e] *Mother's karyotype was 46,XX,t(3;15)(26.2;p11.2)*

tect such a change. The ability to identify quantitative changes of loci using MLPA is dependent upon relative differences between analyzed loci (23). Polyploidy represents an absolute increase of the entire genome and is, as such, not identifiable by MLPA analyses. Other approaches may be considered for the detection of polyploidy (i.e., flow cytometry) (33). If these limitations are considered, it is obvious that an important segment of chromosome changes will be missed if the MLPA analysis is not accompanied by karyotyping.

In cases of contamination of maternal cells, in which a mosaic karyotype was detected by cytogenetic analyses (Table 2), the MLPA results suggested a male karyotype. It is likely that contamination of maternal cells was minimal or below the level of detection by the MLPA method. In one case, a female karyotype was observed after cytogenetic analyses, whereas MLPA confirmed a chromosome Y–positive karyotype. This observation was probably due to live maternal cells besides the non–viable fetal male cells in the tissue sample. Only the maternal cells grew, and therefore the culture was karyotyped as a normal female. Such discordant observations point to a possible problem of tissue culture in cytogenetic analyses: an *in vitro*–induced change can influence the final result. MLPA analyses are not affected because genomic DNA extracted before the tissue culture is used. Therefore, MLPA could be used to clarify the status of certain suspicious abnormalities detected by cytogenetic analyses.

A successful tissue culture is essential to karyotype fetal tissues from terminated pregnancies. This is not always achievable because the available tissue might no longer be viable (e.g., due to asphyxia) or fetal cells with a major chromosome abnormality may not grow readily in culture (34, 35). This problem has been addressed using other methods capable of detecting aneuploidy without the need for tissue culture (14, 22, 33). In the present study, cytogenetic analyses could not be carried out in 18 out of 71 samples because there was growth of fetal cells was absent. With MLPA analyses, we could detect an additional 4 cases of aneuploidy, which were confirmed by CGH (Table 2; Figures 3 and 4). Consequently, a major shortcoming of cytogenetic analyses, i.e., culture failure, has been resolved by MLPA analyses. Combination of the two methods may represent a sensible approach for handling such cases by first screening with MLPA, then independent confirmation of abnormal findings. We used CGH even though other methods are also available. Consequently, the present study indicated that numeric chromosome aberrations (trisomies, monosomies) could be reliably detected by MLPA if both subtelomeric chromosome–specific probes exhibited similar quantitative differences (Figure 1). This criterion was sufficiently stringent to identify all detectable abnormalities and to eliminate false–positive results.

The results of the present study suggested that MLPA analyses could be used to detect nearly all the important chromosome abnormalities present in fetal tissues from terminated pregnancies that would be found by cytogenetic analyses with the notable exception of polyploidy. Therefore MLPA is suitable as a rapid and sensitive supplementary technique to cytogenetic analyses. Recently developed and increasingly popular microarray–based methods are quickly becoming invaluable tools for cytogenetic analyses (20, 21, 22). The amount of information about chromosome structure obtained by MLPA analyses is not comparable with the wealth of data that can be provided by microarray–based techniques (e.g., array CGH). However, MLPA is a 24–h procedure and the setup is simple, with all the necessary chemicals being included in the commercial kits. In addition, the necessary equipment is identical to that required for QF–PCR. Hence, any laboratory offering QF–PCR for rapid detection of aneuploidy could also readily deploy MLPA analyses. Finally, the cost of MLPA analyses is significantly lower than the cost of microarray– based methods.

In conclusion, MLPA analyses using subtelomeric probe sets from all chromosomes can be used to detect numeric chromosome abnormalities and unbalanced structural changes that involve subtelomeric regions. Although MLPA analyses cannot be used to identify the entire spectrum of aberrations detectable by cytogenetic analyses, they can provide substantial information on the structure and quantity of chromosomes. In addition, MPLA can overcome the limitations of karyotyping that result from problems with tissue culturing. The procedure is simple to set up, relatively inexpensive to operate, and easy to integrate into the workflow of a routine genetic laboratory.

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