CEOG Clinical and Experimental Obstetrics & Gynecology

QF-PCR and MLPA: a reliable molecular system to detect chromosomal alterations in miscarriages

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Summary

Purpose of investigation: The aim of this study was to assess the efficacy of the quantitative fluorescent-polymerase chain reaction (QF-PCR) and multiplex ligation-dependent probe amplification (MLPA) combined system to detect chromosome alterations in miscarriage products, as an alternative to conventional cytogenetic testing. *Material and Methods*: This study was conducted between 2011 and 2015 on 264 samples, analyzed using the combined system: QF-PCR/MLPA. This approach first analyzed miscarriage products for chromosomes 13, 18, 21, X and Y, using QF-PCR analysis; in case of ovular fragments, an analysis of maternal DNA was carried out in order to establish the origin of material. Whenever fetal origin was determined, MLPA analysis on the subtelomeric regions was carried out. *Results*: On 264 miscarriages analyzed, 229 were of fetal origin and produced the following results: 53.7% normal and 46.3% pathological. Of the latter, 74.4% were autosomal aneuploidies, 10.4% triploidies, 8.5% sex chromosomal aneuploidies, 3.7% structural alterations, and 2.7% multiple aneuploidies. Results from QF-PCR were obtained from all samples, whereas unambiguous MLPA results were obtained in about 90% of all cases. *Conclusion*: This approach results being highly effective for examining all chromosome aneuploidies, triploidies, as well as structural unbalanced alterations in the subtelomeric regions.

Key words: Recurrent miscarriages; QF-PCR, MLPA; Aneuplodies; Chromosomal alterations.

Introduction

Miscarriage, the spontaneous loss of a clinically established pregnancy before the fetus has reached viability, is the most recurrent complication in the first trimester pregnancy and occurs in approximately 15% of cases [1, 2]. The most frequent causes of pregnancy loss include: anatomical factors, genetic causes (chromosomal anomalies), infections, as well as metabolic and immune disorders. Chromosomal alterations are responsible for about 50% of miscarriage, and are mainly represented by aneuploidies or rearrangement [3]. These alterations are the result of errors during gametogenesis, fertilization or embryonic development and their detection helps to assess the risk of miscarriage for future pregnancies [4].

Cytogenetic testing is the gold standard for identifying chromosomal aneuploidies, polyploidies, and structural chromosome abnormalities, yet it fails in a high rate of cases, because of poor chromosome preparations, culture failure, bacterial or fungal infection of the sample, and maternal cell contamination (MCC) [5, 6]. In the last few years, to overcome these obstacles, different molecular approaches such as quantitative fluorescent-polymerase chain reaction (QF-PCR), multiplex ligation-dependent probe amplification (MLPA) or comparative genomic hybridization (CGH) have been used [4, 6-17].

Among the aforementioned methods, QF-PCR and MLPA have been taken into consideration for their reliability, reproducibility of results, and their low cost. The aim of this study was to assess the efficacy of this combined system to detect chromosome alterations.

Materials and Methods

The Regional Center of Medical Genetics at the University of Perugia, in Perugia (Italy), received materials from a total of 264 recurrent miscarriages (≥ two) from the Obstetrics and Gynaecology Centers throughout the region of Umbria between 2011 and 2015.

Samples consisted of ovular fragments (150 cases) and fragments of skin biopsies (114 cases); gestations varied between five and 21 weeks and maternal age ranged from 18 to 45 years. Data on maternal age, gravidity, parity, number of live births, gestational age, and previous abortions were collected in a database by the Authors. In most cases of the early age miscarriages, ≤ 12 weeks, samples were accompanied by maternal blood in an EDTA tube, in order to exclude maternal cell contamination.

Chorionic villi were isolated from ovular fragments, biopsy skin fragments were cut into small pieces, and these materials were pelleted and washed twice in phosphate buffered saline solution. Maternal DNA was obtained from 200 µl of peripheral blood. DNA extraction was performed using QIAmp Mini and Blood Mini Kit, according to the manufacturer's instructions. DNA was quantified using a biophotometer.

QF-PCR was performed using the system "chromosome aneuploidies QF-PCR kits"; it is a CE-IVD multiplex PCR assay, Cy5-labelled, which amplifies separately six microsatellite

Revised manuscript accepted for publication January 19, 2016

markers for each of the 13, 18, and 21 chromosomes. Regarding sexual chromosomes, the system contains four X-specific markers, two non-polymorphic sequences (AMXY on chromosome X and Y, SRY on chromosome Y), locus X22 mapped on psuedoautosomal region PAR2 (Xq/Yq), and locus TAF9L, mapped on chromosomes 3 and X, the latter which allows to perform an accurate X chromosome dosage. PCR products were separated and the results were analyzed using the CEQ8000 Genetic Analysis System.

P036 and P070 subtelomeres MLPA kits were used according to the manufacturer's instructions, PCR products were separated using CEQ8000 Genetic Analysis System and data were analyzed using the Coffalyser.net platform. These subtelomeres kits contain one probe for the short arm and one for the long arm of each chromosome, with the exception of the acrocentric chromosomes, where a pericentric long arm probe is used. SALSA MLPA Kit Telomere Follow Up Set were used for investigating specific subtelomeric regions. Additional short tandem repeats (STRs) on different chromosomes were used for in-depth investigations.

Results

A total of 264 samples were analyzed using the combined system: QF-PCR/MLPA. Results from QF-PCR were obtained from all samples, whereas unambiguous MLPA results were obtained in about 90% of all cases. QF-PCR allowed the authors to establish the origin of the material, especially in the cases of ovular fragments. A total of 35 cases were excluded from the study as they showed maternal cell contamination or resulted being normal females with no available source of maternal DNA. Thus, the present results refer to a total of 229 cases having fetal origin.

The combination of the two systems allowed the authors to establish that 123 cases (53.7%) were normal and 106 cases (46.3%) were pathological. In Table 1 the pathological results, the relative incidence, the maximum gestational age in which they occurred, and the identification mode are shown. QF-PCR allowed for the identification of 61.3% of pathological results, MLPA of 31.1%, and the combination of the two systems led to identification of the remaining 7.6%. STRs analysis was used in cases of suspicion of triploidy by QF-PCR in order to exclude a multiple aneuploidies involving chromosomes 13, 18, 21, X, Y, and to validate the results.

Regarding involvement, chromosome 21 was the most represented, followed by chromosome 18 and chromosome X, also found in later weeks. As well, aneuploidies involved chromosomes 15, 16, and 22 for a total of roughly 20% of cases found in the early weeks. In some cases of multiple miscarriages in the same woman, aneuploidies involving different chromosomes or different alterations were identified (data not shown). This case type needs to be studied in the future in order to see if there might be a genetic cause associated with these imbalances. In Figures 1 and 2, examples of pathological results and the identification mode are shown.

Table 1. — *Pathological results, their incidence, the maximum gestational age in which they occurred, and the identification mode.*

	Cases	Incidence (%)	Maximum gestational age (week)	QF-PCR	MLPA
Autosomal aneuploidies					
Trisomy 21	25	23.6%	21 w	+	C
Trisomy 18	12	11.3%	14 w	+	C
Trisomy 16	9	8.5%	11 w	-	+
Trisomy 15	8	7.6%	9 w	-	+
Trisomy 13	8	7.6%	20 w	+	C
Trisomy 22	4	3.8%	9 w	-	+
Monosomy 21*	4	3.8%	10 w	+	+
Trisomy 9	2	1.9%	10 w	-	+
Trisomy 2	1	0.9%	8 w	-	+
Trisomy 7	1	0.9%	9 w	-	+
Trisomy 8	1	0.9%	8 w	-	+
Trisomy 10	1	0.9%	11 w	-	+
Trisomy 11	1	0.9%	9 w	-	+
Trisomy 12	1	0.9%	7 w	-	+
Trisomy 20	1	0.9%	7 w	-	+
Sex chromosome aneuploidies					
Monosomy X	7	6.7%	20 w	+	C
XYY	1	0.9%	21 w	+	C
XXX	1	0.9%	n.a.	+	C
Polyploidy					
Triploidy	11	10.4%	18 w	+	-
Structural imbalance					
Deletion 18p, duplication 18q*	2	1.9%	11 w	+	+
Duplication 8p, deletion 15q	1	0.9%	8 w	-	+
Partial trisomy 21 * (region 21q21.1-22.3)	1	0.9%	11 w	+	+
Multiple aneuploidies					
Trisomy 9,trisomy 22	1	0.9%	6 w	-	+
Trisomy 16, trisomy 22	1	0.9%	9 w	-	+
Trisomy 16, trisomy 21,XXX*	1	0.9%	6 w	+	+

Legend: + the system is able to identify the alteration; - the system is not able to identify the alteration; C the system confirmed the result; * both methods are needed to obtain comprehensive and correct results.

Discussion

It has been estimated that in about 41% of cases, spontaneous miscarriage has a genetic cause: the first being autosomal aneuoploidies (~75%), followed by polyploidies (~13%), monosomy X (~8%), and structural imbalances (~4%) [8]. The detection of genetic alterations is fundamental for evaluating of any risk of miscarriage recurrence in future pregnancies.

Cytogenetic analysis is currently recommended for detecting numerical and structural chromosomal abnormalities, yet it often fails because of issues inherent to spontaneous miscarriages, including culture failures, maternal cell contamination which leads to an overestimation

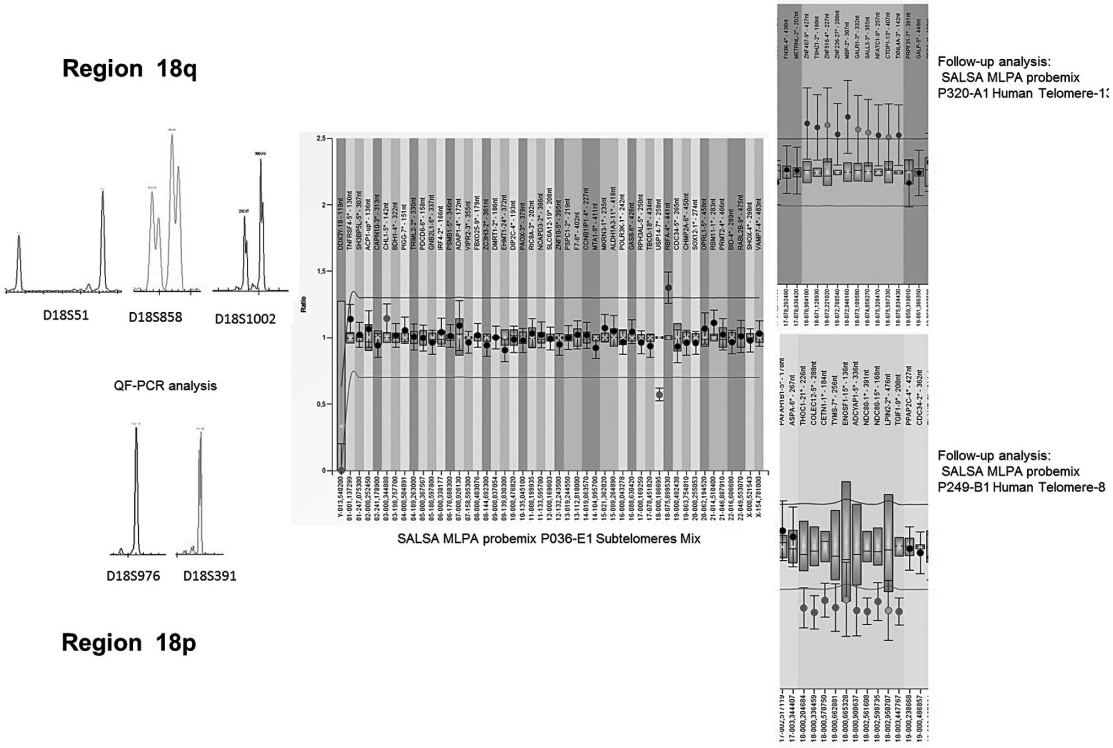

Figure 1. — Deletion on the 18p arm and duplication on the 18q arm. QF-PCR shows a triallelic pattern for STR on 18q and a monoallelic pattern for STR on 18p. MLPA analysis with subtelomers and follow-up kits shows a duplication on the 18q arm and a deletion on the 18p arm.

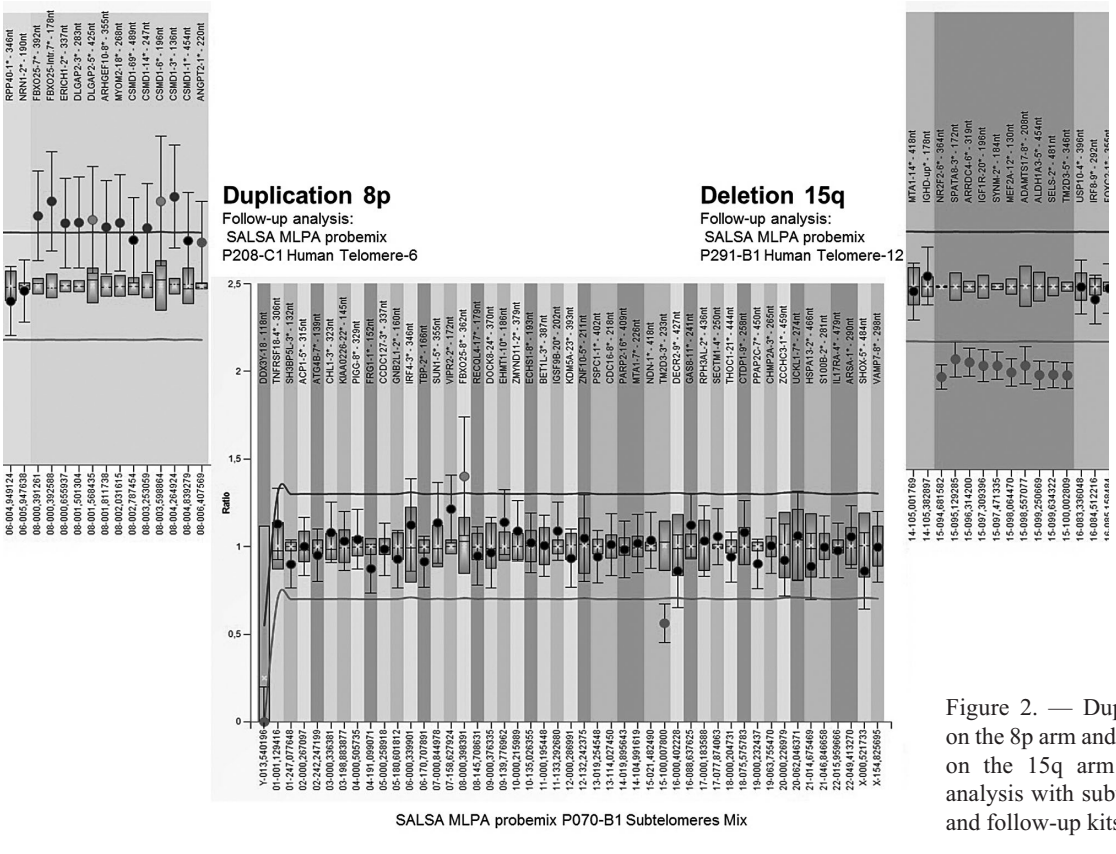

Figure 2. — Duplication on the 8p arm and deletion on the 15q arm: MLPA analysis with subtelomers and follow-up kits.

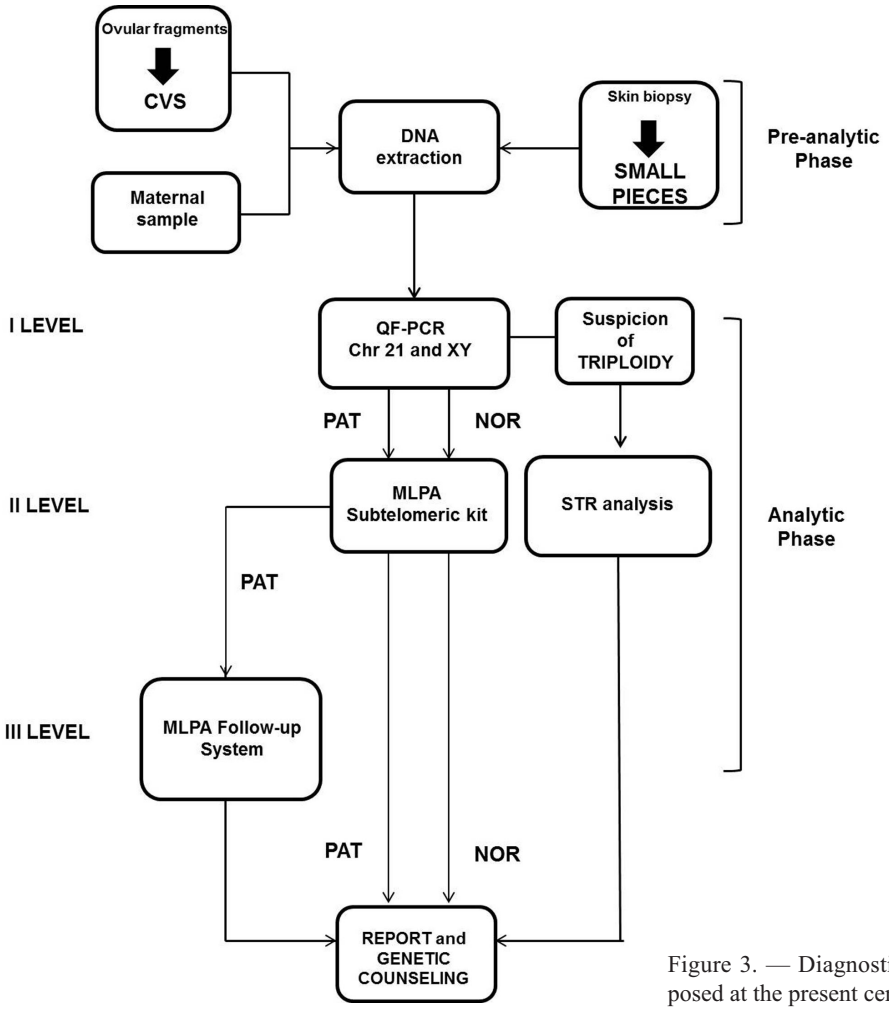

Figure 3. — Diagnostic workup for miscarriage products proposed at the present center.

of false normal female karyotype, as well as bacterial and fungal infections [5, 6].

In the last few years, to overcome these obstacles, different molecular approaches have been proposed. In this study the present authors assessed the efficiency of the combined system, QF-PCR and MLPA, which use genomic DNA extracted from miscarriage products, to detect chromosome aneuploidies, triploidies, and structural unbalanced alterations in the subtelomeric regions, instead the tetraploidies and chromosome mosaicism could not be detected. The choice to use QF-PCR and MLPA was based upon their reliability, reproducibility of results, and their low cost.

This approach first analyzed miscarriage products for chromosomes 13, 18, 21, X, and Y using QF-PCR analysis; in case of ovular fragments, an analysis of maternal DNA was carried out in order to establish the origin of material. Whenever fetal origin was determined, MLPA analysis on the subtelomeric regions (P036/P070) was carried out.

QF-PCR results were obtained for all samples, whereas unambiguous MLPA results were observed in about 90% of cases; this is because the MLPA technique is sensitive to the quality of DNA and therefore it is not always possible to obtain operable DNA samples from miscarriage products.

The QF-PCR analysis determined the origin of the material, identified the most frequent aneuploidies and triploidies, leading to a detection rate of 61.3%. Specifically regarding the cases of triploidy, a rapid and accurate response is necessary, as a hydatidiform mole might be present. When a partial hydatidiform mole due to a paternal origin of the extra haploid set (diandry) is present, the gynaecologist will need to perform additional tests. QF-PCR analysis has fast response-time, no more than 48 hours, and is able to determine the origin of the extra haploid set.

The MLPA analysis not only confirmed the QF-PCR results except for the triploidies, but it was also useful in the following situations: in 30 cases it identified autosomal aneuploidies in chromosomes different from those investigated by QF-PCR; in seven cases of monosomy X, it confirmed conclusions of QF-PCR analysis; in four cases of autosomal monosomy, it defined the results obtained with

QF-PCR: the presence of only one allele for each STR on one chromosome often raises the suspicion of monosomy, but the probability that they are all homozygous is not equal to zero. So, MLPA, which is a quantitative assay that measures chromosomal copy numbers, was able to remove any doubt regarding the presence of an autosomal monosomy; in three cases it leads to a better understanding and completeness of the QF-PCR results: one case of trisomy 21 was a partial trisomy 21 which was not involved in 21q11.2 pericentric region. In two cases QF-PCR results lead the authors to suspect trisomy 18, initially (STR located on the p arm were monoallelic, whereas STR on the q arm were triallelic); the MLPA analysis, using subtelomeres and follow-up kits (SALSA MLPA probemix P320-A1 Human Telomere-13, ten probes on 18q arm; SALSA MLPA probemix P249-B1 Human Telomere-8, ten probes on 18p arm) defined the deletion on the p arm and a duplication on the q arm (Figure 1).

Concerning multiple aneuploidies, in one situation MLPA completed the results obtained by QF-PCR, because the chromosomes investigated by QF-PCR were also involved; in two situations MLPA identified alterations in chromosomes different from those investigated by QF-PCR. One case identified a double structural alteration: a duplication on the p arm of chromosome 8, as well as a deletion on the q arm of chromosome 15. Using MLPA follow-up kits (SALSA MLPA probemix P208-C1 Human Telomere-6, 13 probes on 8p arm; SALSA MLPA probemix P291-B1 Human Telomere-12, ten probes on 15q arm), the authors were able to confirm these results and establish that the alteration involved at least 74.5 Mb on 8p and 5.3 Mb on 15q. (Figure 2).

Considering the results obtained and the chromosomes involved, the authors propose the diagnostic workup shown in Figure 3 for future use in their laboratories. A first screening using QF-PCR on chromosomes 21 and XY, to evaluate the most frequent aneuploidies, the possible presence of triploidy and to define the origin of the material (I level). When triploidy is suspected, other STR on additional chromosomes need to be analyzed in order to lead to a correct diagnosis. The second level, using a MLPA kit for subtelomere regions for all cases of fetal origin (except for triploidies), to confirm either the previously obtained QF-PCR positive results on chromosomes 21 or XY or identify aneuploidies involving other chromosomes, these latter positive results are confirmed using MLPA Follow Up system (III level).

Whenever pathological results are obtained, a genetic counselling to determine whether it is necessary to perform in-depth investigations on parents is recommended, in order to determine any possible risk of recurrence in future pregnancies.

Conclusion

This combined molecular approach of QF-PCR and MLPA is a simple, rapid, and sensitive diagnostic model to identify most of the chromosomal alterations in miscarriages, as alternative to conventional cytogenetic testing.

Acknowledgments

The authors wish to thank Dr. Paolo Prontera and Dr. Carmela Ardisia for their medical support and suggestions.

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