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CLINICAL ARTICLE

Quantitative fluorescent polymerase chain reaction to detect chromosomal anomalies in spontaneous abortion

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Abstract

Objectives: To evaluate the value of short tandem repeats (microsatellites) in the study of numerical chromosomal anomalies in spontaneous abortion. *Method:* Multiplex quantitative fluorescent polymerase chain reaction (QF-PCR) was carried out on 61 spontaneous abortion samples and 48 controls using microsatellite markers from 8 chromosomes where aneuploids are commonly found. *Results:* Of the 61 samples, 65.6% were successfully karyotyped, and the call rate of the QF-PCR was 98.3%. The correspondence between PCR and karyotyping was 95%. The success rate of karyotyping in the inevitable abortion group was 79.6%, higher than for the missed abortion group (8.3%), $P<0.001$. The call rate of QF-PCR showed no difference between these 2 groups (100% vs 91.7%, $P=0.197$). *Conclusion:* Microsatellite-based QF-PCR is a helpful and reliable tool to diagnose numerical chromosomal anomalies in spontaneous abortion. It also provides a diagnosis for necrotic tissue.

1. Introduction

Early pregnancy losses (less than 12 weeks of gestation) affect 12%–15% of clinically recognized pregnancies and up to 60% of all conceptions [1]. Many factors can cause embryo loss, but it is well recognized that 50%–75% of early spontaneous abortions result from chromosomal abnormalities [2–4]. In particular, trisomy causes a large number of spontaneous abortions [5]. Women who have undergone one

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or more spontaneous abortions caused by aneuploidy are at increased risk for chromosomal abnormalities in future pregnancies [6].

Quantitative fluorescent polymerase chain reaction (QF-PCR), based on the analysis of microsatellite markers (short tandem repeats [STR]), is a new diagnostic pathway to detect numerical chromosomal anomaly. The aim of the present study was to assess whether the QF-PCR technique can serve as a complementary method in cytogenetic studies of spontaneous abortion.

2. Materials and methods

Tissues from 61 early spontaneous abortions (at 6 to 12 weeks of gestation, mean age of the patients 30.4±4.2 years) were collected from the outpatient clinic of Shanghai First Maternity and Infant Hospital. If the static sonographic image of the embryo sac corresponded to the expected size according to the calculation based on the date of the woman's last menstrual period, it was defined as inevitable (fresh group) abortion, otherwise it was defined as missed spontaneous abortion (necrosis group). All tissues were handled according to the standard chorionic villus karyotyping procedure on the day of operation. Forty-eight blood samples from unrelated healthy persons (50% male) with a mean age of 35.2±5.44 years from Shanghai were used as controls. Informed consent was obtained from all the individuals tested, and all procedures were carried out with the approval of the local Experimentation Ethics Committee (Department of Obstetrics and Gynecology, Tongji University).

Seventeen microsatellite markers from 7 autosomes and 2 from sex chromosomes (X) were chosen from the literature or the website http://www.gdb.org (Table 1). All 7 chromosomes are most probably linked with numerical anomalies. One amelogenin gene marker [7] was used for differentiating gender. The forward primers were 5′-labeled with the fluorescent dye 6-carboxyfluorescein (FAM). Overall 8 multiplex PCR assays were designed (Table 1). PCR was performed in a total volume of 5 μL containing 10 ng of genomic DNA, 5 pmol of each primer, 2 mM $MgCl_2$, 200 μM dNTPs and 0.25 units of Hotstar Taq (Qiagen; GmbH, Hilden, Germany) on a thermal cycler 9700 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) under a touchdown PCR procedure. All the samples that failed in the first amplification were included in a second procedure through a 10-μL volume system with all the amounts of inner reaction reagents doubled. One microlitre of PCR product was denatured and loaded onto MegaBACE1000 (Amersham Biosciences, Buckinghamshire, UK). The software Genetic Profiler was used for the genotyping of markers.

The ratio of the fluorescent activity for 2 peaks in the PCR products of normal heterozygote (disomic diallelic) was expected to be 1:1. A few cases of disomic monoallelics only showed 1 peak. When referring to a trisomic patient, the STR markers can be detected either as 3 fluorescent peaks, 1:1:1 (complete heterozygote) or 2:1 (trisomic diallelic). The condition of all 3 alleles being coincidently homozygotic is rather rare and could be settled by choosing 2–4 or more markers. In practice, a phenomenon called preferred amplification occurs, meaning 1 of the 2 alleles is amplified first, which acts as a template in the later process, so the actual peak ratio of one disomic diallelic cell should be larger than 1:1 [8].

Genotyping data of the 48 normal control samples were combined to calculate heterozygosity (H) by the formula $H=N^*[1-\sum pi^2]/(N-1)$ [9] (pi is the allele frequency, N is the number of samples) and the peak areas ratio of 2 alleles. The successful karyotyping outcome was used as a blind control to

Table 1 Primers used in multiplex PCR assays

Assay	Marker	Allele Length	Nuclei Repeats	Forward Primer	Reverse Primer
1	D13S174	0.188	DI	5′- FAM GACGACTAACCTCAAGTGCG	TGAAGGCAGAAGTAAAACCATTATC
	D14S80	0.132	DI	5′-FAM-CATCTACCTGCCGCAA	TAGCCAATTTATGGATACAACTT
2	D14S288	0.196	DI	5′-FAM-AGCTAGACTCTGCCATAAACA	TGGAGACAGGAACAACACAC
	D15S127	0.165	DI	5′-FAM-GGGGAACCTACACTTCCG	CCAGGAATCTCAAATGGCTT
3	D21S11	0.172–0.264	TETRA	5′-FAM-GTGAGTCAATTCCCCAAG	GTTGTATTAGTCAATGTTCTCC
	D18S1161	0.082	DI	5′-FAM-GTCCGTCCAACGTCCAA	GGAGAGCCACACCTATCCTG
4	D18S474	0.124	DI	5′-FAM-TGGGGTGTTTACCAGCATC	TGGCTTTCAATGTCAGAAGG
	D21S1270	0.187	TETRA	5′-FAM-CCCACTGTATTATTCAGGGC	ACACACACACACACACATGC
5	D13S162	0.182–0.202	DI	5′-FAM-GCAATCTGAAACATTCTCCA	TTTCATCACAGATAATGCATGATAC
	D13S170	0.113–0.137	DI	5′-FAM-TTGCACTGTGGAGATAAACACATAG	TCACATTGTCTTTTAAGGCAGGAG
	D18S68	0.27–0.290	DI	5′-FAM-ATGGGAGACGTAATACACCC	ATGCTGCTGGTCTGAGG
6	D15S1007	0.165	DI	5′-FAM-GGGGAACCTACACTTCCG	CCAGGAATCTCAAATGGCTT
	D22S1171	0.126	DI	5′-FAM-GAATCACCAGGATGGGCTC	TAGAAGCATTGCCCCGTC
	D21S1411	0.239	TETRA	5′-FAM-ATGATGAATGCATAGATGGATG	AATGTGTGTCCTTCCAGGC
7	D16S3017	0.189–0.197	DI	5′-FAM-TGTCTTATTGGAAGTGTCACCTC	TACAGCCTCTGGCAAATGTT
	D16S539	0.157	TETRA	5′-FAM-GATCCCAAGCTCTTCCTCTT	ACGTTTGTGTGTGCATCTGT
	D22S1157	0.233	DI	5′-FAM-ACCAGCCTGTAGTCCCA	CCAGCATAGAGCAGACATTT
8	DXS8090	0.154	DI	5′-FAM-GGGTGAAATTCCATCACAAA	ACAAATGCAGATGTACAAAAAATA
	DXS8094	0.225–0.239	DI	5′-FAM-GCCATTGTAAAATAAAATTCAG	ATGGTCTTGAGTCACTGTCT
	AMXY*	106, 110		5′-FAM-CCTGGGCTCTGTAAAGAATAGTG	R-ATCAGAGCTTAAACTGGGAAGCTG

The number of the Assay column represents the STR markers combination in multiple PCR. In the Nuclei Repeats column, Di means dinucleotide, TETRA means tetranucleotide. AMXY*, also called Amelogenion gene, is not an STR but can be used to help differentiate gender.

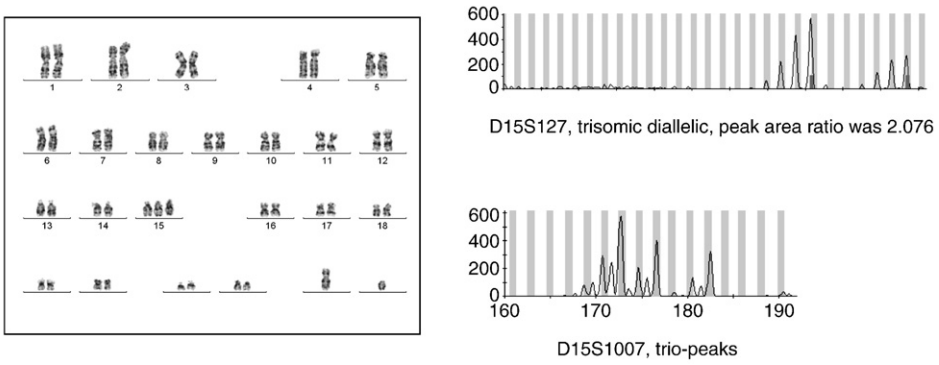

Figure 1 Karyotyping and QF-PCR results for an abortion trial. A trisomy 15 was detected by the two methods. The amplification of D15S1007 showed trio-peaks and the peak area ratio of D15S127 was 2.076 (over than 1.85), corresponding to the diagnosis of trisomy.

evaluate the diagnostic accuracy of the QF-PCR method. Differences between the success rates for the tests were compared using the χ^2 test.

3. Results

Forty of the 61 chorionic villus tissues of abortion samples (65.6%) were successfully karyotyped. Thirteen female fetuses (46,XX) and 11 male fetuses (46,XY) with normal chromosomes were detected. The abnormal karyotypes included 3 cases of trisomy 22; 2 of trisomy 45, XO; 2 trisomy 13; 2 trisomy 18; 2 trisomy 15; 1 trisomy 21; 1 trisomy 14; a 47,XXY; a triploidy; and a mosaicism 46,XX/46,XY. The success rates were 79.6% for the inevitable abortion group and 8.3% for the missed abortion group ($P<0.001$).

According to the genotyping of the 48 normal controls, we found that all of the 19 microsatellite markers had high heterozygosities. The genders of the 48 samples were correctly diagnosed by the 3-marker combination of AMXY, DXS8090, and DXS8094. According to the range of the peak areas ratio for the normal controls, we established that if 2 peaks of a PCR product were within the range of 1.0–1.8, it should be regarded as disomic diallelic, and if the product was found with 3 different peaks or a pattern of 2 peaks with a ratio of more than 1.85, it should be diagnosed as trisomic diallelic. Karyotyping and QF-PCR of a trisomy 15 are shown in Fig. 1.

The QF-PCR call rate of 61 abortion samples was 98.3%. Only 1 sample failed after being amplified twice. Twenty-two samples were found to be numerically anomalous, accounting for 36.1% of the 61 samples. Only 2 cases showed a different outcome than the diagnoses of karyotyping, and the corresponding rate of PCR to karyotyping was 95.0% (59/61). In 1 case, the karyotyping result was 46 XX; the PCR presented irregular peaks after 2 amplifications. The PCR outcome of the other case resulted in normal male peaks, whereas the karyotyping showed a 46XY/46XX. The PCR success call rate was 100% in the inevitable abortion group and 91.7% in the missed abortion group ($P=0.197$).

4. Discussion

Recently many authors have utilized STR-PCR in the prenatal examination of amniocentesis samples and in maternal plasma even after ectopic pregnancy [10–13], but few have incorporated it in the study of spontaneous abortion material [14]. Most STRs have a high level of heterozygosis, and have a low rate of recombination, thus STR markers are widely used in forensic medicine and molecular laboratories [15,16].

In our study, we selected 19 STRs with high heterozygosity to make sure that they were reliable for the diagnosis of chromosomal anomalies and set up diagnostic criteria of a trisomic diallelic sample with a peak ratio over 1.85 according to the data for the control group. The high diagnostic correspondence rate of 95% between QF-PCR and karyrotyping shows that the STR-PCR method is a reliable method that matches the classic cytogenetic method very well. However, we found that QF-PCR was incorrect for mosaicism. These discrepant results may be due to the percentage of mosaicism and the presence of only one of the two cell lines at a detectable level in the test material [17].

The higher successful PCR call rate in the missed abortion group demonstrates that the STR-PCR method can be used on stale tissues; karyotyping strictly requires fresh samples.

The amelogenin gene, with different length of introns, is a pair of alleles located around the kinetochore of the sex chromosome. The difference in the length of the amplified fragment allowed us to determine the gender of a genomic sample [7]. All 48 DNA samples from the controls were correctly diagnosed by the amelogenin gene amplification technique.

Autosomal trisomy accounts for most chromosomal numerical anomalies. It is estimated that chromosomes 15, 16, 14, and 22 are often involved in early spontaneous abortions affected by chromosomal anomalies, whereas chromosomes 13, 18, and 21 and the sex chromosomes are more commonly found in late spontaneous abortions, so the STR markers covering 7 autosomes and the sex chromosomes were chosen for our study [18]. Our cytogenetic findings did not suggest the numerical anomaly that occurred in other autosomes beyond those chosen, supporting the fact that the 19 STR markers and one amelogenin marker are suitable for the diagnoses of common chromosome anomalies with generally accepted high incidence of aneuploidies.

The classic cytogenetic technique has considerable disadvantages: a long laboratory cycle, labor intensive, and culture failure, especially when karyotyping necrotic tissues. Compared with the molecular method and other newly

applied techniques such as fluorescence in situ hybridization, primed in situ labeling, and spectral karyotyping, the STR-based QF-PCR technique is regarded as a highly accurate, low cost, and rapid diagnostic method to detect chromosome numerical anomalies. Osborne et al. [19] established a QF-PCR system to detect chromosome aneuploidy from as few as 10 cells. This method provides us with a supplementary pathway to determine chromosomal anomalies [20]. Ogilvie [21] and Leung et al. [22] recently proposed replacing conventional prenatal cytogenetic analysis with QF-PCR after positive screening for Down syndrome.

However, the technique has limitations; it cannot offer a correct diagnosis for mosaicism, translocation and other small deletions, or duplications [23]. Therefore, the QF-PCR technique will remain a supplementary method to cytogenetic karyotyping for some time.

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