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Research Article

Detection of Fetal Aneuploidies by QF-PCR in Transcervical Cell Samples

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Objective. To evaluate the accuracy in the diagnosis of aneuploidies of a quantitative fluorescent polymerase chain reaction (QF-PCR) assay on trophoblastic cells recovered from transcervical cells samples (TCCs) collected by intrauterine lavage (IUL). *Study Design*. DNA analysis was performed on cells of seemingly trophoblastic origin isolated from IUL samples collected prior to first trimester termination of pregnancy. The analysis was performed by multiplex QF-PCR, using a panel of 29 polymorphic short tandem repeats (STRs) for the chromosomes X, Y, 21, 13, and 18. *Results*. The QF-PCR analysis on placental samples revealed that among the three cases studied there were two cases of trisomy 21 and one case of monosomy X; the comparison of peak profiles obtained from IUL, placental, and maternal samples confirmed the diagnosis of aneuploidy in all three cases. *Conclusion*. This study suggests that the detection of chromosomal aneuploidies in micromanipulated TCC samples can be achieved by QF-PCR amplification of selected highly polymorphic and chromosome specific markers. With respect to standard karyotype, QF-PCR analysis has the limitation that only numerical abnormalities of selected chromosomes can be detected but retains the advantages of being quicker, less expensive, and less lab demanding.

1. Introduction

Currently chorionic villus sampling (CVS) and amniocentesis are the main methods for prenatal genetic diagnosis. Both these procedures are invasive and have been associated with a risk of pregnancy loss of 1% [1]; also, cells need to be cultured for the obtainment of a karyotype, and two weeks are commonly required before the results can be made available to the pregnant woman. A further problem with amniocentesis is that the technique is performed at 16–18 weeks' gestation, and this results in the late termination of affected pregnancies with a more traumatic method and an evident psychological distress.

For these reasons the attainment of prenatal genetic diagnosis by mini-invasive techniques has been extensively studied in recent years; both fetal cells and cell-free DNA have been found in the maternal circulation, but clinical implications are limited thus far to the prediction of fetal sex and Rh status [2, 3].

In a different line of research it has been attempted to detect trophoblastic cells in transcervical cell (TCC) samples. Following the pioneering study by Shettles [4] who first claimed he could accurately predict fetal sex by simply observing the "Y-body fluorescence" on quinacrine-stained mucus samples collected from the cervix, many groups have reported on the successful prediction of fetal sex and prenatal genetic diagnosis using trophoblastic cells from TCC samples. Overall the results of these studies show very different figures as to the rates of correct sex prediction and genetic diagnosis, and this is probably due to the diverse techniques used both for sample collection and analysis. Indeed, TCC sampling may involve either the collection of the mucus plug (by cytobrush or by aspiration) or the flushing of the lower genital tract at various levels (endocervix, uterine cavity), and it is now clear that these procedures do differ in terms of trophoblastic cell yield, since the reported rates of correct sex prediction are 24–70% for mucus samples [5, 6] and 75–91% for intrauterine lavage samples [7, 8]. With respect to the methods used for the analysis, an important limitation of most previous studies is that they were simply intended for demonstrating the presence of trophoblastic cells within the samples, and an aim pursued using either fluorescence

in situ hybridisation (FISH) or PCR-based assays to detect Y chromosome sequences in samples from male fetuses [5]. However, if the clinical use of these samples for prenatal genetic diagnosis is concerned, trophoblastic cells must not only be detected but also isolated in view of subsequent genetic analysis; in this respect, recent studies by our group show that intrauterine lavage (IUL) samples, unlike mucus samples, often contain trophoblastic cells to such an extent to enable easy detection and isolation [9–11].

In recent years, the availability of the quantitative fluorescent polymerase chain reaction (QF-PCR) assay has contributed greatly to the investigation of TCC samples for prenatal diagnostic purposes. The QF-PCR technique relies on the amplification of chromosome-specific short tandem repeat (STR) sequences using fluorescent primers, whereas the analysis of the products of amplification is performed using an automated DNA sequencer. Usually STRs with high degree of polymorphism are used, and more than one STRs for each single autosomal chromosome are investigated at any one time in order to achieve informative results on the chromosome copy number; while the lack of highly polymorphic markers had hindered the detection of sex chromosome abnormalities until a few years ago, in recent times many of these markers have been identified, and the QF-PCR analysis of all numerical sex chromosome disorders has finally became possible [12].

The aim of the present investigation was to evaluate the accuracy in the diagnosis of aneuploidies of a QF-PCR assay on trophoblastic cells recovered from TCCs collected by IUL.

2. Materials and Methods

We analysed three IUL samples collected prior to first trimester termination of pregnancy (TOP), requested for increased aneuploidy risk following nuchal translucency measurement. None of the women had undergone an invasive prenatal diagnostic procedure before TOP. All the women gave their informed consent.

With the woman in the lithotomy position, under general anesthesia, a speculum was inserted to visualize the cervix which was disinfected with an iodine solution, and IUL was then performed using a 2.5 mm inner diameter flexible catheter (Nelaton Catheter; Medinorm, Quierschied, Germany) attached to a syringe filled with 10 mL of sterile saline solution. The catheter was carefully inserted through the cervical canal, just past the internal os; the saline was instilled under gentle pressure and then 2–6 mL (mean value 3.5 mL) were aspirated back after 10 seconds.

Shortly after collection, IULs were transferred into a Petri dish and observed under inverted microscope: cells clumps and villous filaments of seeming trophoblastic origin (Figure 1) were isolated using a pipette with sterile-plugged tip, washed in sterile saline solution, and cut in a separate dish with 2 mL of sterile saline solution. From each sample a maximum of 10 mg of villi were prepared for DNA extraction.

A fragment of placental tissue was collected at TOP in all cases, as well as a sample of peripheral blood from the mother. Placental tissue samples were also observed under an inverted microscope to select chorionic villi (about 10 mg) for DNA extraction.

2.1. QF-PCR Procedure. DNA was extracted from the micromanipulated IUL, from placental tissue, and from 200 uL of peripheral blood samples, using an automated system (Biorobot EZ1, QIAGEN SpA, Milan, Italy), with a DNA yield of 15–20 ug for tissues and up to 8 ug for blood. For the QF-PCR analysis of the chromosomes X, Y, 21, 18, and 13, a panel of 29 STR markers (listed in Table 1) were selected on the basis of their highly polymorphic nature.

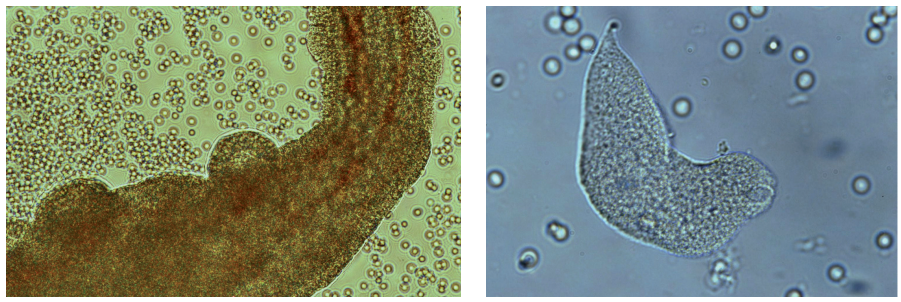
Fetal sex was assessed using the nonpolymorphic sequence of the amelogenin region (AMXY), which corresponds to two different specific products for the X and the Y chromosomes (104 bp and 109 bp, resp.); two additional sex chromosome markers were used: the pentanucleotide repeat X22, which maps to pseudoautosomal region (PAR2) of the X and Y chromosomes and the X-linked hypoxanthine phosphoribosyltransferase (HPRT), amplified together with the D21S1411 as an autosomal internal control for its quantification, to distinguish between a normal female fetus homozygous for all X markers and a monosomy X fetus [13].

All reactions were set up in a final volume of 25 uL containing 10 ng of DNA, 200 umol/L dNTP, 2–25 pmol of primer, 2 mmol/L $MgCl_2$ in 1x Taq buffer, and 1 U of Taq polymerase (AmpliTaq Gold, Applied Biosystems, Foster City, CA, USA). After denaturation for 2 min at 95°C (hot start procedure) the samples were subjected to 28 cycles of amplification (35 s at 94°C, 1.30 min at 58°C, 1 min at 72°C, plus a final extension of 15 min at 72°C) in a programmable thermal controller machine (PTC-100, MJ research, Watertown, MA, USA). One uL of 1 : 10 diluted QF-PCR products, mixed to 12 uL of formamide and 0.3 uL of size standard (TAMRA 500, Applied Biosystems, Foster City, CA), was separated, after 3 min of denaturation, in an ABI Prism 310 automated genetic analyser (Applied Biosystems, Foster City, CA, USA). All forward primers were fluorescently labelled for the assessment of the size and the amount of the PCR products. Analysis of the results was performed with GeneScan software 3.1 (Applied Biosystems, Foster City, CA, USA).

3. Results

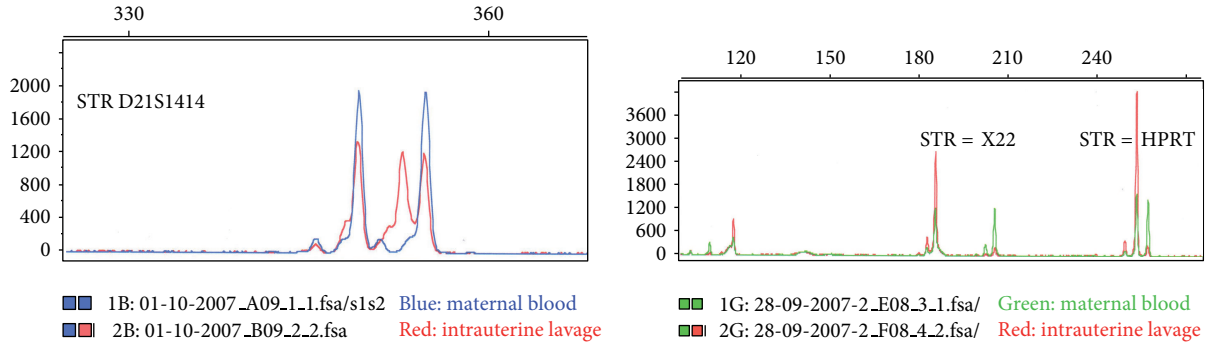
In all three cases chorionic villi and/or cells clumps of seeming trophoblastic origin could be detected and sorted by micromanipulation under inverted microscope, at which time the samples were also checked for possible sperm contamination that could be ruled out in all cases.

The QF-PCR analysis on placental samples revealed that among the three cases studied there were two cases of trisomy 21 and one case of monosomy X; the same findings were documented also in micromanipulated IULs, where the peak pattern obtained precisely matched that of the corresponding placental samples, thus confirming the trophoblastic origin of the cells selected from IULs (Figure 2). In addition, the comparison of peak profiles obtained from IUL, placental, and maternal blood samples was useful to clarify the inheritance of the various STR alleles used in



(a) (b)

Figure 1: Villus (a) and trophoblastic cell clump (b) from IUL observed under inverted microscope.



(a) (b)

Figure 2: (a) Electrophoretogram showing the comparison of maternal and fetal peaks after amplification of DNA for the STR marker D21S1414. Triallelic pattern in a fetus with trisomy 21. (b) Monosomy X resulting from paternal nondisjunctionin the IUL there is only one allele (inherited from the mother) for each of the two selected STRs (X22 and HPRT).

the study. For instance it can be seen (Figure 2(b)) that in the case of monosomy X the single allele present in the fetus for each of the X-related STRs was present also in the mother, thus strongly suggesting paternal nondisjunction as the causative event of the aneuploidy; conversely, in the case of trisomy 21 presented in Figure 2(a) two out of the three alleles of the STR D21S1414 are found in the mother, and this finding (corroborated also by data not shown, relative to all the other STRs specific to chromosome 21) implies that trisomy resulted from maternal nondisjunction.

4. Discussion

In recent years earlier and safer alternatives to amniocentesis and CVS have been pursued. Ideally, fetal or trophoblastic cells should be obtained early in pregnancy by a safe procedure, be easily detected and isolated and then analysed by a reliable method. The presence of trophoblastic cells in TCC samples has been repeatedly confirmed in most studies, although there are relevant and sometimes striking differences as to the rate at which trophoblastic cells are found, depending also on the methods used for sample collection. Indeed, TCCs have been collected by a number of different procedures involving either cervical mucus sampling or the flushing of the lower uterine pole [5, 14], and it has been argued that while trophoblasts could be present in the mucus plug as a result of either exfoliation or active migration [5], IUL might rather act as a mini-CVS [6]. This could explain in some measure the much higher rates of trophoblastic cells in IULs, as compared with mucus samples, that had been put forward in earlier studies and have finally found confirmation in a recent investigation aimed at comparing the two techniques in the same group of women [6]. IULs always contain a mixture of cells from the maternal genital tract, and the problem of selecting only trophoblasts for further genetic analysis has been overcame by performing micromanipulation under inverted microscope, a simple and undemanding procedure that yields trophoblastic elements in more than 80% of all IULs [9].

Regarding the methods for analysis, the introduction of the QF-PCR assay in prenatal diagnosis has represented a relevant advance since its use on both CVS and amniotic fluid samples allows the quick and reliable detection of numerical chromosomal abnormalities [12]. QF-PCR could give also a major contribution to the possible use of TCCs for the purposes of prenatal diagnosis; as a matter of fact, this approach enables the precise assessment of chromosome copy number in IULs from both male and female fetuses, since, unlike FISH and standard PCR amplification techniques, it relies on the analysis of DNA polymorphisms and can therefore

Table 1: STR markers, with extension and position, used in the study.

Marker	Label	Heterozygosity	Chromosome location	Known alleles in bp
AMXY	6-fam	—	Xp22.1-22.31-Yp11.2	X 104 Y 109
SRY	6-fam	—	Yp11.2	Y 463
X22	6-fam	0.91	Xq28 Yq (PAR2)	189-194-199-204-209-214-219-224-226-229-234-239-242-247-253
DXYS218	PET	0.65	Xp22.32 Yp 11.3 (PAR1)	266-270-274-278-282-286-290-294
HPRT	6-fam	0.75	Xq26.1	268-272-276-278-280-284-288-292-296-300-313
DXS6803	VIC	0.68	Xq12-Xq21.33	106-110-114-118-120-124-128
DXS6809	VIC	0.75	Xq	238-242-246-250-252-254-258-260-262-266-268-270-274
DXS8377	NED	0.85	Xq28	213-216-219-222-225-228-238-241-244-248-252
SBMA	NED	0.75	Xq11.2-Xq12	166-169-172-175-178-181-184-187-190-193-196-199-202-205-208-211
D21S1414	6-fam	0.85	21q21	328-330-334-338-342-346-350-352-354-356-358-360-362-443
D21S1411	VIC	0.93	21q22.3	246-262-266-274-278-282-286-290-294-298-302-306-316-319
D21S1446	PET	0.77	21q22.3-ter	200-204-208-212-214-218-220-224-228
D21S1437	PET	0.78	21q21.1	120-124-128-132-136-140-144
D21S1008	6-fam	0.70	21q22.1	196-200-204-208-212-216-220
D21S1412	6-fam	0.73	21q22.2	384-388-392-396-400-406-410-414-418
D21S1435	VIC	0.75	21q21	142-168-172-176-180-184-188
D18S391	VIC	0.75	18pter-18p11.22	144-148-152-156-160-164-168
D18S390	VIC	0.75	18q22.2	398-402-406-410-414-418-422-426-430
D18S535	NED	0.82	18q12.2	126-130-134-138-142-146-148-152-156
D18S386	NED	0.89	18q22.1	319-330-334-338-342-344-350-354-358-362-366-370-372-376-380-387
D18S858	PET	0.66	18q21.1	186-190-192-196-200-204
D18S499	6-fam	0.72	18q21.32-q21.33	386-392-396-400-404-408
D18S1002	6-fam	0.80	18q11.2	122-130-134-138-142
D13S631	VIC	0.78	13q31-32	192-196-200-204-208-212-215-218
D13S634	VIC	0.85	13q14.3	460-464-466-470-474-478-482-484-486-490-496-500
D13S258	NED	0.89	13q21	230-232-234-236-238-240-242-244-248-265-267-269-271-273-277-279-281
D13S305	PET	0.75	13q12.1-13q14.1	426-430-434-438-442-446-450-454-458
D13S628	6-fam	0.70	13q31-q32	436-440-444-448-452-456-460-464
D13S742	VIC	0.75	13q12.12	254-258-262-266-268-270-274

discriminate the maternal and trophoblastic origin of the cells.

The results of this study on the one hand confirm that IUL samples are a valuable source of trophoblastic cells that can be easily isolated [10] and on the other hand suggest that aneuploidies such as trisomy 21 and monosomy X, that account for a remarkable proportion of all clinically relevant chromosomal disorders, can accurately be detected in these cells by a QF-PCR assay. Our results are important, as, in spite of the body of research on TCCs, very little had been previously reported on the detection of chromosomal or genetic disorders using these cells [15–19], which is no doubt a crucial point in view of the possible clinical use of the procedure.

A limitation of the approach described in this study, and in particular of the QF-PCR analysis, is that only numerical abnormalities of selected chromosomes can be detected, but only standard karyotype enables full evaluation of all numerical as well as structural alterations. This drawback of the QF-PCR procedure is, however, outweighed by many advantages such as quickness of results and minor lab costs. Although there is a preliminary study on continuing pregnancies reporting no complications following IUL [20], further research is required to precisely assess the safety

of this sampling technique; in an ongoing study IUL is being performed in a group women scheduled for TOP at the time of first booking in order to have a short yet significant period of followup for possible procedure-related complications.

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