ORIGINAL INVESTIGATION

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Non-invasive early prenatal molecular diagnosis using retrieved transcervical trophoblast cells

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Abstract Fetal DNA was recovered from 17 of 39 (44%) transcervical cell (TCC) samples obtained between 7 and 9 weeks of gestation by endocervical canal flushing. Trophoblast retrieval was adequate for polymerase chain reaction (PCR) amplification of Y chromosome-specific DNA sequences and detection of paternal-specific microsatellite alleles. The fetal sex predicted by PCR in TCCs was confirmed in all cases by karyotype analysis of chorionic villi at 10 weeks of gestation. The absence of the disease-associated paternal alleles in TCC samples from two pregnancies at risk for spinal muscular atrophy and myotonic dystrophy predicted unaffected fetuses in agreement with subsequent results on chorionic villi and newborns' leukocytes. A trisomy 21 fetus was diagnosed in TCCs using fluorescent in situ hybridization (FISH) and semi-quantitative PCR analysis of superoxide dismutase-1 (SOD 1). Present experience indicates that TCC sampling is a promising technique for early prenatal monitoring of Mendelian disorders and chromosome aneuploidy.

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Introduction

Diagnostic methods currently used in prenatal diagnosis of genetic diseases are hampered by the slight risk of miscarriage related to the invasivity of these procedures (Brambati 1993). Recent studies have reported also an association between chorionic villus sampling (CVS) and fetal limb abnormalities (Holmes 1993; Firth et al. 1994). Although these findings are not conclusive, nor corroborated by the largest retrospective study, there is a quite general agreement to perform CVS at 10–11 weeks of gestation (Froster and Jackson 1994). A few non-invasive approaches to the prenatal diagnosis of genetic diseases have been proposed in recent years. Most of these protocols are based on isolation of fetal cells from the maternal blood in combination with ultrasonography and maternal serum analyte analysis (Adinolfi 1992; Simpson and Elias 1994; Ewigman et al. 1993; Muller et al. 1993). In addition, experimental data have been obtained on the collection and analysis of fetal cells using fluorescence activated cell sorter (FACS), magnetic activated cell sorter (MACS), specific monoclonal antibody, fluorescent in situ hybridization (FISH), and polymerase chain reaction (PCR) methods (Simpson and Elias 1993; Zheng et al. 1993). However, further investigations are needed to validate the large-scale applicability and effectiveness of these methods. Retrieval of trophoblast cells from the lower part of the uterine cavity has been proposed as a source of fetal DNA obtainable by a non-invasive procedure (Griffith-Jones et al. 1992). In fact, from 6–8 weeks of gestation, degenerating trophoblast cells shed into the endocervix, where they can be collected for genetic analysis (Shettles 1971; Warren et al. 1972). Early experience has demonstrated accurate fetal sex determination in transcervical swabs between 9 and 13 weeks of gestation (Shettles 1971), while inconclusive results were reported in other studies (Morris and Williamson 1992).

Informed consent was obtained and transcervical cell samples (TCCs) recovered between 7 and 9 weeks of gestation from a consecutive series of 39 women requesting

first trimester prenatal diagnosis for advanced age according to standard CVS protocols at 10 weeks of gestation. TCCs were examined by phase-contrast microscopy to evaluate the quantity and quality of the retrieved material. FISH and a semi-quantitative PCR analysis of superoxide dismutase 1 (SOD 1) gene copies were carried out in TCCs to evaluate the possibility of performing prenatal diagnosis of trisomy 21. Molecular investigations included PCR amplification of the Y chromosome in male-bearing pregnancies, and analysis of paternal-specific microsatellites. TCCs were also used for monitoring two pregnancies at risk for spinal muscular atrophy type I, (SMA I; MIM*253300) and myotonic dystrophy (DM;MIM*160900).

Materials and methods

TCC samples were collected at 7–9 weeks of gestation from 39 women undergoing first trimester prenatal diagnosis for advanced age, including two pregnancies at risk for SMA I and DM. Patients were informed of the experimental nature and purpose of the study and a written informed consent was obtained from all women entering into the research project. TCCs were recovered under ultrasound control by flushing of the lower uterine cavity and the endocervix using a commercially available flexible plastic tube. The tube was connected to a syringe containing 2–3 ml of physiological saline which was instilled and retrieved for analysis. After collection, aliquots of the cells were examined by phase-contrast microscopy and electron microscopy. Additional samples were prepared for conventional FISH analysis (Baldini and Ward 1991).

FISH analysis

FISH was performed using routine techniques with biotin- or digoxygenin-labeled probes specific for chromosome 21 (chromosome contig 21, Cambio, UK) and chromosome 2 (alpha-satellite, D2Z, Oncor, USA). About 60–100 cells were examined, and image analysis was performed as previously reported (Baldini and Ward 1991). The results of FISH tests were compared with those obtained from cytogenetic analysis of CVS cells.

DNA isolation and PCR amplification

DNA was extracted from TCC, blood and chorionic villi samples using a standard phenol:chloroform extraction procedure. The concentration of each DNA sample was adjusted to 50 ng/µl using double-distilled sterile water. Sex determination was performed by PCR amplification of fragments of the testis-determining gene (SRY, 254 bp) and the human sperm receptor expressed in the human zona pellucida, ZP_3 (177 bp) according to Cui et al. (1994). The D21S11 locus was amplified as described by Mansfield (1993). The D5S127 and D5S663 loci were amplified and analyzed according to Lo Cicero et al. (1994). Semi-quantitative PCR detection of trisomy 21 was performed using two sets of primers flanking exon 5 of the SOD 1 gene on chromosome 21 (Deng et al. 1993) and exon 7 of the cystic fibrosis transmembrane regulator gene (CFTR) on chromosome 7 (Zielenski et al. 1991). The two sequences were simultaneously amplified by standard methods except for the addition of [^{35}S]-dATP to the reaction. Products were resolved in thin 4% polyacrylamide gels which were exposed to autoradiography at –70° C for 1 week. Results were expressed as a ratio of the signals from SOD 1 and CFTR products determined by densitometry using Molecular Dynamics ImageQuant software. DNA from pregnancy at risk for the DM mutation was digested with *Eco*RI, electrophoresed in a 0.8% agarose gel and transferred onto nylon membranes (Hybond N, Amersham). Filters were probed with MDY-1 which contains the variable CTG repeat (Novelli et al. 1993).

Results

Microscopy analysis

Cytologic examination of TCCs by a phase-contrast inverted microscope revealed the presence of villi sproud in 16 of 39 samples (41%) and isolated single syncitiotrophoblast cells in 21 of 39 (Fig. 1). Additional cell types found in all samples were squamous cells, neutrophils, and lymphocytes. The number of recovered maternal and fetal cells was highly variable in different samples, although in the present series no definite evaluation could be established between time of sampling and failure in obtaining fetal cells. A systematic analysis of slides failed to detect any spermatozoa in the total series of TCCs.

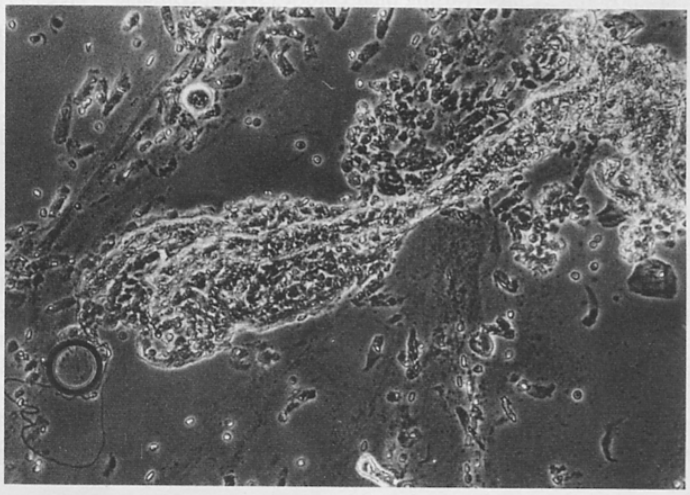

Fig. 1 Invertoscope view of a chorionic villi sproud (10X) collected by transcervical cell *(TCC)* sampling

Y-specific PCR and microsatellite analysis

In order to identify fetal DNA in TCCs, PCR sex determination and paternal-specific alleles were investigated on DNA extracted from transcervical samples. Parallel studies were carried out in parental blood and CVS collected at 10 weeks of gestation. Informativity was defined by the presence in TCCs of either the SRY-specific PCR amplified products in male fetuses or paternally derived microsatellite alleles at the D21S11, D5S127, and D5S663 loci. Seventeen of the 39 TCCs analyzed (44%) showed detectable fetal DNA. Seven male fetuses were predicted by PCR detection of the SRY fragment and confirmed by molecular analysis and sex karyotyping of chorionic villi at 10 weeks of gestation (Fig. 2). PCR sexing did not provide any false-positive results. Distinct informative alleles were identified in the ten fetuses with negative SRY PCR amplification, either at the three loci analyzed and with concordant results, or at one of the three loci, indicating the presence of the paternal allele. The relative intensity of bands in the mixed samples suggested that fetal DNA was less than ¼ of the total DNA. This figure was estimated by comparing the TCC results with those obtained by diluting 10–1000 times in vitro the DNA extracted from chorionic villi and maternal blood. In 22 samples no paternal microsatellite allele nor Y-specific amplification signal was detected, suggesting that the recovered TCCs were maternal in origin. PCR was carried out also on DNA retrieved from cervical and vaginal swabs in 6 of the 39 pregnancies in which samplings were performed in different areas of the genital tract. This allowed assessment of the specificity of the results in samples containing fetal DNA (Fig. 3). All transcervical specimens contained fetal DNA, while positive results were obtained in cervical and vaginal swabs only in 2 of 6 pregnancies.

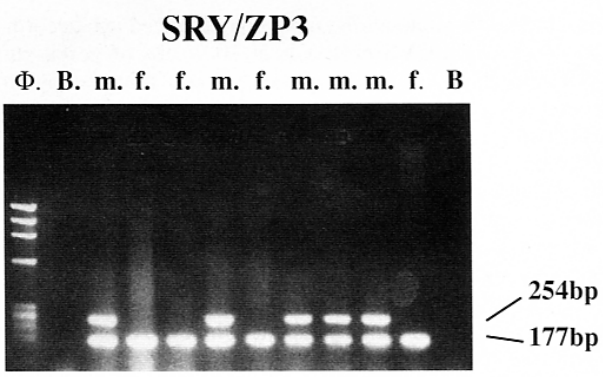

Fig. 2 PCR sexing in a series of TCC samples. SRY-specific sequence amplification (254 bp of the testis-determining gene) and ZP3 internal control (177 bp). (*Φ* DNA molecular weight marker ΦX174/*Hae*III, *B* negative control, *m* male fetus, *f* female fetus)

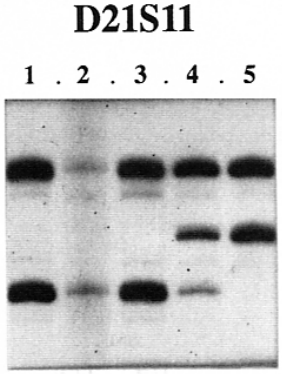

Fig. 3 PCR amplification of the D21S11 marker in DNA extracted from maternal blood (lane 1), vaginal swabs (lanes 2, 3), endocervical sampling (lane 4), and chorionic villus sampling (*CVS;* lane 5)

Prenatal diagnosis

All TCC samples containing fetal DNA were examined for chromosome 21 ploidy using dual-color FISH experiments in interphase nuclei. Sixty to 100 nuclei were examined from each slide, with a hybridization efficiency of over 80%. Trisomy 21 was detected in a TCC sample flushed from a 43-year-old women at 7 weeks of gestation (Fig. 4). This result was confirmed at 10 weeks by direct cytogenetic analysis of CVS. FISH results were also corroborated by semi-quantitative PCR analysis in the total DNA extracted from TCCs. The co-amplification of exon 5 of the SOD 1 gene (chromosome 21 test) and exon 7 of the CFTR gene (target sequence from chromosome 7) after 31 PCR cycles revealed that the SOD 1 amplified product was approximately 1.5 times higher in trisomy 21 TCC DNA compared to euploid cells (data not shown). This result was validated also in CVS DNA. Two pregnancies at risk for SMA I and DM were monitored by TCCs analysis. The family at risk for SMA I had been genotyped previously for the microsatellite markers (Lo Cicero et al. 1994). The family at risk for DM had been examined for the presence of CTG expansion which was present in the affected father (Novelli et al. 1993). As shown in Fig. 5, the fetus at risk for SMA I was predicted to be unaffected because the TCCs showed that the paternal allele at the D5S127 locus (allele 2) was different from the allele segregated in the affected child. Also, the fetus at risk for DM was predicted to be unaffected, based on the presence of normally sized alleles in TCCs DNA (Fig. 6). In both pregnancies the TCC result was confirmed on chorionic villi at 11 weeks of gestation and further validated at birth.

Discussion

It has been shown that fetal cells are present in the cervical canal of pregnant women (Griffith-Jones et al. 1992; Pertl et al. 1994a), where they can be collected by flushing the mucus from the canal with a physiological solution and used for cytogenetic and molecular analyses (Griffith-Jones et al. 1992; Pertl et al. 1994b; Chaouat et al. 1994; Ishai et al. 1995). To date, only a few studies have supported the diagnostic value of this procedure (Adinolfi et al. 1993; Bernini et al. 1994). Adinolfi et al. (1993) have

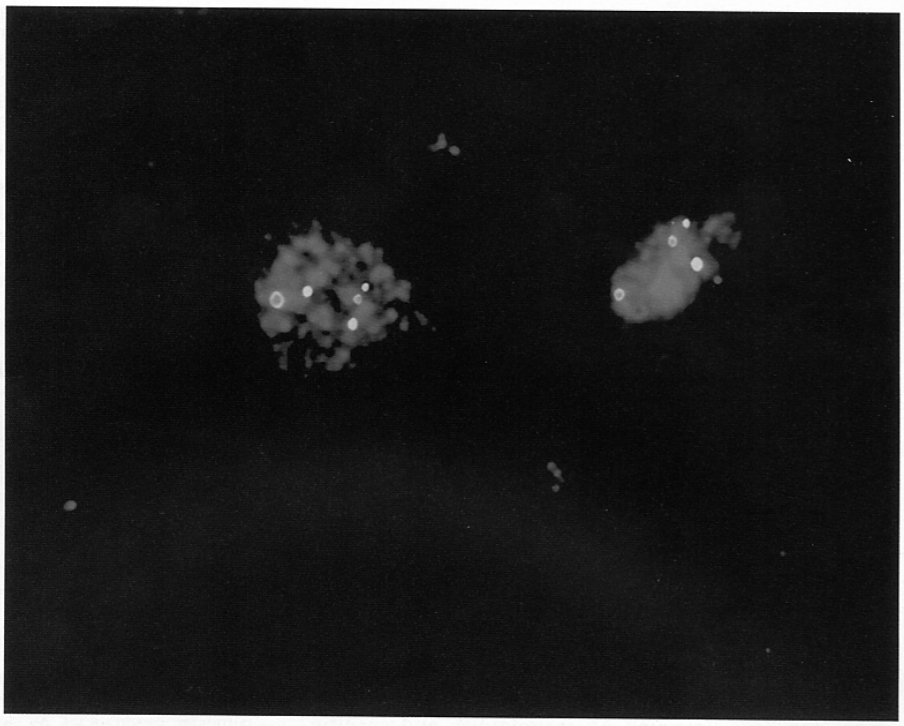

Fig. 4 Detection of trisomy 21 in a TCC sample using a dual-color fluorescent in situ hybridization *(FISH)* experiment on interphase nuclei. Biotinylated chromosome 21-specific probe *(white spots)*, digoxigenated chromosome 2-specific probe *(red spots)*. The fetal cell at the left shows three white spots, while the maternal cell at the right shows two white spots

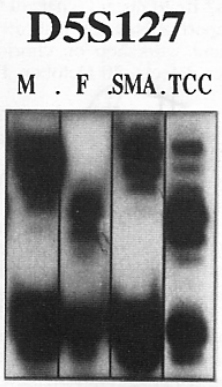

Fig. 5 Spinal muscular atrophy *(SMA)* exclusion in a fetus at risk using TCCs analysis of marker D5S127. (*M* mother DNA genotype 1/3, *F* father DNA genotype 2/3, *SMA* affected child DNA genotype 1/3, *TCC* transcervical cell DNA genotype 1/2/3, showing the presence of paternal wild-type allele 2 and the maternal alleles 1/3)

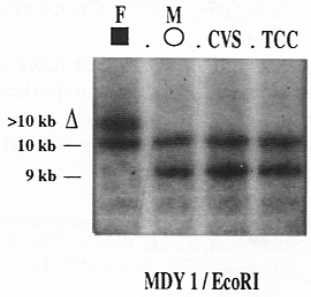

Fig. 6 Myotonic dystrophy *(DM)* exclusion in a fetus at risk using TCC analysis of a CTG-specific probe (pMDY1). (*F* DM-affected father, *M* mother, *CVS* chorionic villi, *TCC* transcervical cells)

detected trisomy 18 and Y-derived sequences using FISH and primed in situ (PRINS) labelling techniques in TCCs. However, doubts were raised on the results obtained on TCCs retrieved after CVS which could have introduced iatrogenically apparent fetal cells in the lower uterine pole (Gaudoin 1993). Nevertheless, immunological and morphological evidence prove that trophoblastic cellular elements are present in the endocervical canal between 8 and 13 weeks of gestation (Pertl et al. 1994a; Adinolfi et al. 1993). Recently, Adinolfi et al. (1995) have detected fetal alleles in 4 of 14 (28%) TCC samples collected by flushing and examined by PCR and FISH. Our study confirms and extends previous results by showing that fetal cells are present in TCC samples from 7 weeks of gestation. In addition, external contamination is ruled out because TCC samplings were performed before CVS in all cases. Sperm contamination has been considered a limiting factor in TCC studies (Morris and Williamson 1992; Chaouat et al. 1994; Zimmerman et al. 1994). We failed to detect any spermatozoa by careful electron microscopic scrutiny of all samples. This result was also corroborated by the concordance of PCR sexing analysis in TCCs and CVS. In addition, maternal contamination complicates the interpretation of molecular results, particularly when the mother is the affected parent or is heterozygous for the same recessive mutation present in the father.

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Another the yield of recovered fetal DNA was low, we have shown that in about 50% of pregnancies this is adequate for PCR amplification of specific mutated paternal alleles and enables identification of single-gene disorders. The failure in detecting fetal DNA in the remaining TCC samples may be related to the low sensitivity of microsatellite analysis, or a difficulty in collecting an adequate amount of trophoblast material. Therefore the sampling success rate requires further technical improvement. Studies are in progress in our and other laboratories (Pertl et al. 1994a) using monoclonal antibodies and chromatographic techniques to achieve this objective.

The present study supports previous results (Pertl et al. 1994a; Adinolfi et al. 1994, 1995) indicating that the TCC sampling technique is promising. However, further experimental investigations are needed to establish the large-scale applicability of this method in early prenatal diagnosis. In particular, the non-invasivity of the TCC sampling technique requires evaluation by long-term follow-up analysis. In order to propose this procedure as an alternative to CVS it is necessary to prove also that TCC sampling can be carried out confidently at a significantly earlier gestational age compared to the standard first trimester sampling procedures. Finally, more experimental data are required to prove the constancy and adequacy of fetal DNA in TCCs for developing quantitative PCR assays for early detection of aneuploidies and specific mutated alleles (Mansfield 1993; Crotty et al. 1994; Pertl et al. 1994b).

The results reported in this paper represent a substantial advance compared to previous experiences and show that the non-invasive first trimester prenatal diagnosis from endocervical samples has the potential to become an alternative procedure.

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