Trophoblast Retrieval and Isolation From the Cervix for Noninvasive, First Trimester, Fetal Gender Determination in a Carrier of Congenital Adrenal Hyperplasia

Reproductive Sciences
2016, Vol. 23(6) 717-722
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DOI: 10.1177/1933719116632922
rs.sagepub.com

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Abstract

Congenital adrenal hyperplasia (CAH) is an autosomal recessive defect in cortisol biosynthesis that elevates fetal androgen levels to cause genital ambiguity and external genital masculinization in newborn females. Introducing dexamethasone in utero by 7 weeks gestation precludes virilization of affected females. However, identification of a male fetus prior to week 7 could avert the necessity of steroid treatment in half of pregnancies at risk of CAH. We recently introduced trophoblast retrieval and isolation from the cervix (TRIC), an approach that noninvasively isolate homogeneous trophoblast cells from pregnant women as early as 5 weeks gestation, using a Papanicolaou test. Here, we have used TRIC to correctly identify male fetal DNA when both parents were carriers of the mutation that produces CAH and previously produced an affected child. Trophoblast cells (1400) obtained by TRIC were assessed using immunocytochemistry with an antibody against the trophoblast-specific β subunit of human chorionic gonadotropin, which labeled 100% (17 of 17) of isolated cells, while none of the excluded maternal cervical cells were labeled. The isolated cells were examined by fluorescent in situ hybridization for chromosomes 18, X, and Y at a clinical cytogenetics laboratory, demonstrating 100% (18 of 18) of cells to be diploid 18/XY. Aliquots of DNA obtained from the isolated cells assayed for SRY and RNASEH genes by TaqMan assays confirmed a male fetus. This case study demonstrates the utility of TRIC to accurately identify fetal gender as a means of reducing the need for prophylactic administration of exogenous steroids in pregnancies at risk of CAH.

Keywords

congenital adrenal hyperplasia, prenatal genetic diagnosis, placenta, trophoblast, fluorescence in situ hybridization, steroids, 21-hydroxylase

Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive defect in cortisol biosynthesis. The incidence of the classical form of CAH is approximately 1:15,000 live births, with a higher risk in certain populations such as Yupik Eskimos, with a frequency as high as 1:282.¹ Clinical consequences arise from both failure to synthesize hormones in the adrenal cortex downstream of the enzymatic deficiency as well as consequences of cortisol precursor accrual.² Faulty conversion of 17-hydroxyprogesterone to 11-deoxycortisol by the enzyme steroid 21-hydroxylase (21-OHD) is attributed to more than 95% of cases with CAH and results in decreased cortisol synthesis.³ 21-Hydroxylase, encoded by the *CYP21A2* gene, is also

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responsible for the conversion of progesterone to deoxycorticosterone in the synthetic pathway of aldosterone. Phenotypes vary depending on the extent of 21-OHD impairment, with the most severe salt-wasting form causing hyponatremia, hyperkalemia, hypovolemia, and even death if left untreated.⁴ Decreased cortisol raises adrenocorticotropin hormone levels, resulting in overproduction and accumulation of 17-hydroxyprogesterone (17-OHP). 17-Hydroxyprogesterone is then shunted to the intact androgen pathway, where 17,20-lyase converts it to androgens. High androgen levels cause genital ambiguity and external genital masculinization in newborn females and gradual postnatal virilization in either sex, unless treated.^{5,6} Screening for CAH is highly effective in decreasing mortality by distinguishing the salt-wasting form.⁷ Dexamethasone treatment in utero, when introduced early in gestation, will preclude virilization of an affected female fetus. Prenatal intervention with dexamethasone for CAH is not without complications.⁸ When steroids were given before 9 weeks to a cohort that included 49 pregnancies, 11 of the 25 female fetuses had normal female genitalia, 11 presented with minimal virilization, and 3 were virilized (Prader stage 3). Despite appropriate pharmaceutical intervention, 15% of the affected female fetuses will accede to virilization.^{9,10}

Treatment to prevent masculinization of external genitalia should preferably be introduced by 5 to 6 weeks of gestation, unless a karyotype of 46, XY is established.¹¹ Molecular genetic analysis of fetal DNA acquired by amniocentesis or chorionic villus sampling (CVS) is used to diagnose CAH in utero.¹ Delaying steroid intervention until the karyotype can be obtained through conventional prenatal diagnostic approaches would not benefit a female fetus. Yet steroids pose risks to the mother and should be avoided when possible. Due to the autosomal recessive inheritance pattern, parents who are both heterozygous for the mutation have a 1 in 8 risk of producing an affected female fetus. Mothers who undergo prenatal dexamethasone treatment will, therefore, be unnecessarily treated in 7 of 8 cases.¹² Preimplantation genetic diagnosis (PGD) provides an option for diagnosis of fetal CAH in couples known to be carriers for the disease.¹³ However, this approach requires in vitro fertilization, which is not available to all couples and commands a high cost. Furthermore, many couples who are carriers for CAH conceive naturally. Cell-free fetal DNA obtained from maternal plasma offers a promising noninvasive approach that relies on complex bioinformatics to distinguish small fragments of fetal DNA from a much greater fraction of maternal DNA.¹⁴ The possibility of diagnosing 21-OHD mutations by massively parallel sequencing of cell-free fetal DNA in maternal blood has been tested with success prior to 7 weeks gestation.¹⁵ The approach requires adequate read depth and genomic coverage to establish the fetal genotype. Currently cell-free fetal DNA is clinically approved for chromosome number determination^{16,17} but is not considered reliable for mutational analysis, which would

require higher base pair resolution.¹⁸ Cell-free fetal DNA accumulates to detectable levels with increasing gestational age and is adversely affected by maternal obesity,¹⁹ which could present a challenge for many pregnant plasma samples taken before the seventh week that contains less than 1.5% fetal DNA. Therefore, patients carrying a fetus at risk of CAH would benefit by knowing fetal gender prior to week 7. Those carrying a male could forego prophylactic steroid therapy, and the remainder could either utilize steroid treatment or seek genetic testing of circulating cell-free fetal DNA.

Reliable DNA testing can be performed with a small number of fetal cells without need for massively parallel sequencing, given the success of PGD with single blastomeres and trophoctoderm biopsy.²⁰⁻²² Intact fetal trophoblast cells are reliably obtained from ongoing pregnancies through safe, noninvasive endocervical collection,²³ preferably with a cytobrush and ThinPrep kit,²⁴ and can be separated from maternal cells using trophoblast retrieval and isolation from the cervix (TRIC).²⁵ Determination of fetal gender after TRIC is straight forward and was exceptionally accurate in 20 consecutive samples analyzed by polymerase chain reaction (PCR) as well as by fluorescence in situ hybridization (FISH). Examination of over 200 specimens collected between weeks 5 and 20 of pregnancy has shown TRIC to reliably provide 500 to 1000 trophoblast cells at purities above 95%, without interference by maternal obesity.²⁶ It is hypothesized that TRIC could accurately identify a male fetus in pregnancies at risk of CAH prior to the time when steroid therapy would be initiated. To support the hypothesis, a clinical case is presented in which prenatal gender testing by TRIC was used in the seventh week of gestation to correctly identify male fetal cells from a pregnancy conceived by parents who previously had a child with CAH and who both harbored mutations in 21-OHD.

Case Study

A 25-year-old gravida 2 para 1001, who presented at the University clinic for an infertility work up, had an unremarkable gynecological history with regular menses and a first menarche at 16 years of age. Her medical history included kidney stones that required lithotripsy and esophageal dilatation for a stricture. Her obstetrical history included a previous normal spontaneous vaginal delivery that was diagnosed with CAH. Genetic analysis of the parents revealed a maternal 30 Kb deletion of *CYP21A2* and a paternal mutation of Exon 1, 5' end c.57 G>A (W19X). The female patient was also a carrier of a Fragile X Syndrome premutation CGG repeat that was determined on 2 separate analyses to have 32 and 55 repeats, respectively. Her husband underwent a vasectomy in 2010 and had a successful microsurgical epididymal sperm aspiration procedure that produced adequate sperm for in vitro fertilization by intracytoplasmic sperm injection. The patient underwent ovarian stimulation, and 15 oocytes were retrieved for in vitro fertilization. Seven embryos had adequate maturation, and

prior to embryo transfer, the embryos were biopsied for PGD. Two male embryos without evidence of the 30 kb deletion of *CYP21A2* were transferred on day 5. A positive β subunit of human chorionic gonadotropin (β -hCG) was obtained, and an ultrasound examination at 7 weeks documented positive cardiac activity. At that time, an endocervical specimen was obtained from the patient for fetal gender analysis by TRIC. The pregnancy continued normally to term with the birth of twin males, indicating that both transferred embryos had gestated successfully.

Materials and Methods

Endocervical Sampling

The Institutional Review Board of Wayne State University approved the study, with written informed consent obtained from the patient prior to participation. Endocervical sampling was conducted as described previously.²⁴ Briefly, the cervical specimen was collected with a ThinPrep kit (Hologic, Inc, Marlborough, Massachusetts), using a cytobrush. The cytobrush was rinsed into 20 mL of PreservCyt (Hologic) fixative solution, acidified to dissolve mucous, and centrifuged at 400 g for 5 minutes at 4°C. The supernatant was removed, and the cell pellet was resuspended in 20 mL of ice-cold sterile phosphate-buffered saline (PBS). Specimens were then washed by centrifugation and resuspension 2 more times in 20 mL PBS. The final wash was brought to 10 mL with PBS at 4°C.

Immunomagnetic Isolation of Trophoblast Cells

The processed endocervical cells were centrifuged and suspended in 1.5 mL of PBS, combined with magnetic nanoparticles (Clemente Associates, Madison, CT) coated with anti-human leukocyte antigen G (HLA-G), and incubated overnight at 4°C with mixing to isolate trophoblast cells, as detailed previously.²⁵ The nonbound cells were collected after magnetic immobilization of HLA-G-positive trophoblast cells. A small aliquot of the isolated cell suspension was removed and counted to determine the number of cells recovered in the isolate. Isolated HLA-G-positive cells were suspended in groups of approximately 50 cells in 200 μ L of PBS and centrifuged onto slides using a Shandon Cytospin 3 cytocentrifuge (Thermo Fisher Scientific, Waltham, MA) at 1500 rpm for 5 minutes. The purity of isolated trophoblast cells was determined by immunofluorescence for the β -hCG. The percentage of β -hCG-positive cells was quantified using secondary antibody conjugated to fluorescein isothiocyanate and a 4',6-diamidino-2-phenylindole (DAPI) nuclear counterstain, as described previously.²⁵

Polymerase Chain Reaction

Real-time fluorescent PCR was used to determine the gender of isolated HLA-G-positive cells. Cells were lysed directly for

PCR, removing aliquots equivalent to approximately 2 cells into a 10- μ L reaction mix containing Mastermix, TaqMan probes, and primers for SRY and RNASEH (Life Technology, Carlsbad, CA). The reaction mixture was incubated for 10 minutes at 95°C, followed by 60 cycles of 45 seconds at 95°C, and 45 seconds at 60°C, using a BioRad (Hercules, CA) CFX 364 real-time fluorescence thermocycler, with automated data analysis.

Fluorescence In Situ Hybridization

Isolated trophoblast cells were analyzed by FISH at the Detroit Medical Center Cytogenetics Laboratory, using the AneuVysion kit (Abbott Molecular Des Plaines, IL) for detection of chromosomes X, Y, 13, 18, and 21, according to the manufacturer's protocol. Briefly, the slides were immersed in 100 mL of 0.01 N HCL containing 11 mg pepsin for 10 minutes at 37°C $\pm$ 1°C. Slides were then washed once in PBS for 5 minutes at room temperature and fixed with in 1% formaldehyde for 5 minutes at room temperature. Slides were then washed in PBS for 5 minutes at room temperature and dehydrated by serial immersion into 70%, 85%, and 100% ethanol at room temperature for 1 minute each. The slides were allowed to dry completely and were hybridized with AneuVysion probe sets (3 μ L of each probe) at a denaturation temperature of 75°C for 5 minutes, followed by hybridization at a temperature of 37°C for 16 to 20 hours. Slides were washed in 0.4 $\times$ saline sodium citrate (SSC) containing 0.3% nonyl phenoxypolyethoxyethanol (NP-40) at 73 $\pm$ 1°C for 2 minutes, then 2 $\times$ SSC/0.1% NP-40 at room temperature for 2 minutes. Cells were counterstained with 3 μ L of DAPI. Using an Olympus BX51 (Tokyo, Japan) epifluorescence microscope equipped with a 120-W OEX bulb, the slides were analyzed using dual orange/green band-pass, single aqua band-pass, and DAPI band-pass filters. Number of signals for chromosomes 18, X, and Y were then counted and recorded for each cell.

Results

Isolation and Purity of Trophoblast Cells

A total of 1400 trophoblast cells were recovered after immunomagnetic isolation. To establish that the isolated cells were trophoblast, immunofluorescence microscopy was used to examine expression of β -hCG. The percentage of immunomagnetically bound cells expressing β -hCG was 100% (17 of 17), while no maternal cells (0 of 68) were labeled with the antibody.

Gender Determination of Isolated Trophoblast Cells

Cells were analyzed by FISH to evaluate the X and Y chromosomes as well as chromosomes 13, 18, and 21. The FISH analysis confirmed both X and Y chromosomes in 18 of 18 cells examined, consistent with a normal male genotype (Figure 1).

Figure 1. Fluorescence in situ hybridization (FISH) for chromosomes 18, X, and Y in trophoblast cells obtained by trophoblast retrieval and isolation from the cervix (TRIC). Trophoblast cells were labeled with probes YP11.1-q11.1 (spectrum orange; red) for the Y chromosome, Xp11.1-q11.1 (spectrum green; green) for the X chromosome, and 18p11.1-q11.1 (spectrum aqua, light blue) for chromosome 18. Nuclear chromatin is labeled with 4',6-diamidino-2-phenylindole (DAPI; blue). Two examples of microscopic fields with labeled cells are shown. Size bar, 25 μ m. (The color version of this figure is available in the online version at <http://rs.sagepub.com/>.)

Additionally, each cell was diploid for chromosomes 13 (10 of 10), 18 (18 of 18), and 21 (10 of 10).

The SRY (male) and RNASEH (autosomal copy number) genes were assayed in fetal cell DNA, using real-time PCR with TaqMan probes. The fluorescent reaction products increased above threshold for both SRY and RNASEH with fetal cell DNA as well as with control human male genomic DNA (Figure 2). The fetal DNA used for PCR was equivalent to approximately 2 cells (~ 12 pg) and generated expectedly higher cycle threshold values (SRY, 39; RNASEH, 42) than the 50 pg of genomic male DNA (SRY, 35; RNASEH, 34). Negative controls lacking DNA produced no signal above background.

Figure 2. Real-time fluorescent polymerase chain reaction (PCR) analysis of trophoblast cells obtained by trophoblast retrieval and isolation from the cervix (TRIC). The change with cycle number in relative fluorescence units (RFU) produced by TaqMan reporter dyes for SRY (blue lines) and RNASEH (green lines) is tracked during the PCR. The thicker horizontal lines indicate RFU thresholds used to determine cycle threshold (Ct) values. The amount of fetal DNA (Fetal DNA) used in the reaction was equivalent to 2 cells isolated by TRIC, while a positive control (Control Male DNA) contained 50 pg of male human genomic DNA. A negative control (Blank) that lacked substrate DNA produced no fluorescence above the threshold after 60 cycles. (The color version of this figure is available in the online version at <http://rs.sagepub.com/>.)

Discussion

Trophoblast cells accumulate in the endocervical canal and can be recovered by a number of methods.²³ The use of a cytobrush to collect cells from the cervix has been shown to be safe in several studies of over 1900 patients.²⁷⁻³¹ Multiple investigators have attempted to use these cells for prenatal genetic diagnosis. However, the approach has been limited by the inability to efficiently separate the fetal cells from the excess of maternal cervical cells. With antibody against HLA-G, a protein present on trophoblast cells and not on maternal cervical cells,³² we have demonstrated the ability to isolate on average 750 trophoblast cells from co-mingling maternal cervical cells with a high degree (>95%) of purity in ongoing pregnancies.²⁵ Furthermore, gender identification by both FISH and PCR is reliable as early as 5 weeks of gestation.^{25,26} In the present case study, we isolated 1400 intact trophoblast cells at 7 weeks of gestation that were homogeneous based on β -hCG labeling. All of the isolated cells exhibited an XY genotype to correctly identify male gender. This case introduces a novel and safe approach for early prenatal determination of fetal gender in pregnancies at risk of CAH or other sex-linked genetic disorders and demonstrates the potential of TRIC for genetic testing earlier in gestation than other available procedures.

Prenatal diagnosis of CAH is clinically significant because in utero treatment is available to circumvent virilization of an affected female fetus. In families with a history of CAH, as exemplified in this case study, PGD is recommended.

Historically, prenatal diagnosis of CAH required analyses of fetal genomic DNA obtained through invasive methods with either CVS or amniocentesis, which at earliest could be performed at 10 and 15 weeks of gestation, respectively, and carry a risk of fetal loss.³³ Androgen excess can virilize a female fetus as early as 6 to 7 weeks of gestation, so prenatal dexamethasone treatment prior to prenatal testing has proven an effective method of limiting virilization.⁴ Dexamethasone, a potent steroid, has side effects in the mother, including excessive weight gain, hyperglycemia, and severe striae.³⁴ The safety of this procedure for children treated in utero remains controversial regarding future cognitive functions.^{9,35} It is important to discontinue therapy as soon as possible in pregnancies with a male fetus or unaffected female fetus. However, waiting until prenatal genetic diagnostic results are available would unnecessarily expose mothers of unaffected offspring to steroids for 6 to 8 weeks.³⁶ Because of these complications, prenatal steroid therapy is considered experimental by the Endocrine Society.¹⁰ If genetic testing to identify unaffected fetuses were available, implementation of early dexamethasone treatment would be better justified.

The decision to withhold early steroid treatment based on gender determination by TRIC should be taken with caution, unless a singleton pregnancy can be clearly established. In the present case study, twin boys were conceived, which was not a problem, since it was known that only male embryos were transferred. In a mixed gender multiple gestation, a female fetus could go undetected in a test for the Y chromosome, although the presence of mixed male and female cells by FISH with a high percentage of β -hCG-expressing cells would be indicative of this possibility. Taking these considerations into account, TRIC gender analysis could prove highly useful as a noninvasive early screen used in conjunction with other confirmatory methods. TRIC is a safe, inexpensive, and relatively simple approach that could contribute to reducing unnecessary steroid exposure during pregnancy in patients at risk of transmitting CAH.

Conclusion

Trophoblast cells accumulating in the endocervical canal and isolated by TRIC offer a novel approach for noninvasive prenatal gender testing as early as 5 weeks of gestation. Patient specimens can be obtained in an office setting as an outpatient procedure, since cells collected into the ThinPrep fixative are stable during transportation to a laboratory and can be stored several days under refrigeration. Gender determination by TRIC would benefit patients at risk of transmitting genetic mutations for CAH who do not have access to PGD or who conceive naturally. Early determination of male fetal gender would prevent unnecessary use of exogenous steroids to prevent masculinization. Because TRIC isolates intact cells with a complete fetal genome, it could potentially be used to detect genetic mutations, such as those that cause CAH. Due to the simplicity of TRIC, this approach could benefit populations lacking access to advanced reproductive technologies. The

isolation of intact trophoblast cells by TRIC will eventually provide opportunities for whole fetal genome analysis and targeted sequencing of medically important genes.

Authors' Note

This work was performed at the Mott Center for Human Growth and Development, Wayne State University School of Medicine.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: MPD, SD and DRA have a pending patent and receive payment for intellectual property that has been licensed on their behalf by Wayne State University to Perkin Elmer, Inc.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Supported in part by the Intramural Research Program of the National Institute for Child Health and Human Development, National Institutes of Health and grants from the NIH (HD071408, HL128628) the W.K. Kellogg Foundation, the March of Dimes Foundation, and Perkin Elmer, Inc.

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