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Potential of syncytiotrophoblasts isolated from the cervical mucus for early non-invasive prenatal diagnosis: Evidence of a vanishing twin

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ABSTRACT

Background: Non-invasive methods to assess the foetal genome during pregnancy will provide new opportunities to offer pregnant women a more comprehensive genetic diagnosis of their established foetus. The aim of this study was to determine the presence and frequency of foetal cells in transcervical cell (TCC) mucus samples from pregnant women and determine their suitability for early prenatal diagnosis.
Methods: Syncytiotrophoblasts in aspirated TCC mucus samples were identified by immunostaining with the foetal-specific antibody NDOG1. Genetic analysis of foetal cells was performed by laser capture microdissection and quantitative fluorescent PCR (QF-PCR).
Results: In 116 of 207 (56%) TCC samples, abundant syncytiotrophoblasts were retrieved. However, when TCC samples were stratified for the presence of chorionic villous fragments, syncytiotrophoblasts were identified in 85 of 109 (78%) samples. Significant numbers of syncytiotrophoblasts were found in TCC samples collected between 6 and 9 weeks of gestation (mean 741, range 25–2884). QF-PCR analysis of NDOG1 positive syncytiotrophoblasts and matching maternal DNA confirmed their foetal origin and correct foetal cell sexing was achieved in 97% of TCC samples. The one discordant sex diagnosis was associated with a dizygotic dichorionic twin pregnancy resulting from the implantation of a female T21 embryo and a normal male embryo, where the female T21 foetus had succumbed at 6 weeks of gestation and was vanishing.
Conclusions: Syncytiotrophoblasts can be successfully isolated from TCC samples and represent a suitable source of cells for genetic analysis of the established foetus in early pregnancy. The study highlights a vanishing twin as a potential cause for discordant non-invasive prenatal test results.

1. Introduction

Trisomy 21 (Down syndrome) is a commonly diagnosed chromosomal abnormality in the human foetus. For example, in the period from 2004 to 2006, the estimated prevalence of Down syndrome in the United States was approximately 14.47 per 10,000 live births, which means that 1 in every ~690 infants born were affected with Down syndrome [1]. The likelihood of having a child with Down syndrome is strongly correlated with maternal age, with the risk increasing in a gradual, linear fashion until about the age of 30 and then exponentially up to the age of 45 [2,3]. Currently, prenatal screening tests are widely offered by antenatal clinics to pregnant women over 35 years of age who are at greater risk. Despite the success of these screening programmes up to 61% of all infants with Down syndrome are born to women under the age of 35 who are not routinely offered testing [4]. By maternal serum screening, in combination with ultrasound assessment of nuchal translucency, approximately 90% of all Down syndrome pregnancies can be detected [5]. However, this screening regimen is still associated with a 5% false positive rate leading to unnecessary invasive diagnostic tests such as chorionic villus sampling and amniocentesis, which exposes the pregnancy to a small (0.6–2%), but significant risk of miscarriage [6]. It is for this reason that couples are reluctant to accept this increased risk especially if the female partner is of advanced reproductive age or has experienced difficulty with achieving a pregnancy.

During the past two decades, research has largely focussed on the development of non-invasive approaches for the diagnosis of chromosomal abnormalities using alternative and more accessible sources of foetal cells or DNA. The dominant technology that has emerged is massively parallel sequencing of the cell free circulating foetal DNA [7] which is rapidly becoming integrated into routine prenatal care [8]. This test focuses on the detection of common trisomies involving such as T21, T18 and T13 as well as sex chromosome aneuploidies that comprise over 50% of the chromosomal abnormalities that persist into

Abbreviations: TCC, transcervical cell; LCM, laser capture microdissection; QF-PCR, quantitative fluorescent polymerase chain reaction; TOP, termination of pregnancy; W, weeks; CV, chorionic villus; PBS, phosphate buffered saline; H&E, haematoxylin and eosin; STR, short tandem repeat; *HPRT*, hypoxanthine guanine phosphoribosyl transferase; AMELX, amelogenin X; AMELY, amelogenin Y; D21, disomic Chr21; T21, trisomic Chr21.

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the first trimester [9]. In prospective studies [10,11], the non-invasive prenatal test for trisomy detection is highly reliable and accurate with sensitivities and specificities for T21, T18 and T13 greater than 99%, and by comparison, out performs current maternal serum screening [12]. Nonetheless, there are some limitations associated with making an accurate diagnosis using the fragmented foetal DNA in the maternal plasma, including the detection of other chromosome disease syndromes associated with smaller copy number variations as well as single gene diseases. Recent studies suggest that deeper sequencing [13] and mutation targeting [14] may eventually provide an effective solution for comprehensive genetic diagnosis of the foetus using cell free foetal DNA, however, further studies are needed to validate these approaches for clinical application.

The ability to reliably isolate small numbers of foetal cells with an intact genome using a non-invasive method would open up new possibilities in the field. Attempts to isolate foetal nucleated red blood cells from maternal blood for genetic analysis has proven difficult due to their rarity (1 to 2 cells per ml of blood) [15], the inefficiency of cell sorting techniques using foetal cell specific antibodies against cell surface markers such as CD71 and variable levels of maternal cell contamination [16]. More recently, using size filtration techniques, small numbers of trophoblastic cells were isolated from blood samples of pregnant women and used to successfully diagnose correct genotypes for foetuses at risk for cystic fibrosis and spinal muscular atrophy [17]; however, the reliability of retrieving sufficient trophoblasts in a larger number of pregnancies requires further investigation. An alternative and more abundant source of foetal cells resides in the cervical mucus of pregnant women [18]. These foetal cells are believed to emanate from the regressing chorionic villi into the lower uterine pole and accumulate behind the mucus at the level of the internal os [19]. Foetal cells in the endocervical canal can be retrieved by transcervical cell (TCC) sampling as early as 5 to 7 weeks of gestation using either endocervical lavage, aspiration or with cytology brushes [20–25]. The availability of a reliable and safe technique would allow the possibility of minimally-invasive early prenatal diagnosis. However issues still remain as to the optimal TCC sampling technique and the availability of robust strategies to isolate pure populations of foetal cells from the vast majority of maternal cells [25].

We previously developed a promising procedure using the foetal-specific antibody NDOG-1 [23] in combination with laser capture microdissection (LCM) [24] to isolate pure populations of foetal cells from TCC samples obtained by catheter aspiration. In a preclinical trial using foetal cells isolated from cervical mucus we further demonstrated 100% accuracy for correct sex determination of the foetus [24]. To further evaluate the clinical potential of our method, we analysed 207 TCC samples to determine the frequency of foetal cells at different gestational ages and test the ability to use LCM purified foetal cells to simultaneously diagnose sex and Down syndrome.

2. Materials and methods

2.1. Collection of TCC samples

The Human Research Ethics Committee at the Monash Medical Centre, Melbourne, Australia approved the TCC sampling procedure and written informed consent was obtained from patients prior to the procedure. A total of 207 pregnant women undergoing elective termination of pregnancy (TOP) between 6 and 9 weeks (W) of gestation consented to the study. The mean maternal age of the patients undergoing TOP was 29 (range, 18–46 years of age). TCC samples were collected using a fine aspiracath (Cook, Australia) by a medically qualified general practitioner skilled in the procedure. Briefly, the catheter was inserted approximately 2–3 cm into the cervix at the level of the internal os and gentle aspiration was applied to collect a TCC sample (mean volume of 0.5 ml, range 0.1 to 1.0 ml). Based on visual examination, TCC samples were very heterogeneous consisting of variable combinations and types of mucus including stringy white fragments, a highly viscous mucus and a solid tissue-like mucus. Microscopic examination of the more frequently occurring mucus with stringy white fragments revealed finger-like structures typical of chorionic villous. Given the heterogeneous nature of the TCC samples with respect to mucus composition, we divided the samples into two comparative study groups based on either the presence of chorionic villous (CV+) or the absence of chorionic villous (CV−) regardless of the presence of other mucus types. A maternal buccal cell sample was also taken for comparative molecular analysis to assist in foetal cell identification. The gestational age of the foetus was determined by either the time of the last menstrual period, an ultrasonographic scan or both.

2.2. Processing of TCC samples

TCC samples were treated in 20 mg/ml L-acetyl cysteine for 30–45 min at 37 °C to dissolve the mucus. Cells were washed in phosphate buffered saline (PBS) and resuspended in 1 ml of PBS to give a homogeneous cell suspension. For each sample, 20 μl aliquots of the cell suspension was spread thinly over the surface of two PEN membrane slides (PALM Microlaser Technologies AG, Bernried, Germany) and over the surface of two Super Frost plus slides (Menzel-Glaser, Germany) and then air-dried. Slides were fixed in 100% ethanol and then stained with haematoxylin and eosin (H&E). The cellularity of the samples was assessed by routine light microscopy and the presence of cells of seemingly trophoblastic origin (multinucleated syncytiotrophoblasts) was determined. Samples were further examined for the presence of other cell types including squamous and endocervical cells, neutrophils, lymphocytes and red blood cells.

2.3. Determination of foetal cell numbers in TCC samples

Forty-three consecutive CV+ samples shown to contain significant numbers of syncytiotrophoblasts by H&E staining and light microscopy were also subjected to immunohistochemistry with a trophoblast-specific antibody to more accurately assess the frequency of syncytiotrophoblasts in TCC samples collected between 6 and 9 W of gestation. The NDOG1 monoclonal IgM antibody (Serotec Ltd, Oxford, UK) was identified as a candidate antibody with foetal specificity as it only positively stains the intervillous syncytial elements; maternal cells including lymphocytes, granulocytes and red blood cells are not stained [19,26–28]. Briefly, slides were washed in 0.05 M Tris pH 7.6, 0.15 M NaCl (TBS). Endogenous peroxidase activity was then blocked with 3% hydrogen peroxide (Merck Pty Ltd, Kilsyth, Australia) in absolute methanol for 2 min, followed by a further incubation with neat normal goat serum (Sigma, Missouri, USA) for 10 min at RT to block non-specific antibody binding. Syncytiotrophoblasts were labelled with NDOG1 antibody followed by secondary labelling with the Universal DAKO Labelled Streptavidin — Biotin®R2 System, Horseradish Peroxidase (DAKO Cytomation, CA, USA). The reaction was developed with liquid DAB substrate-chromogen, ready-to-use (DAKO Cytomation, CA, USA), counterstained with Mayer's Haematoxylin, dehydrated and mounted in synthetic resin, DPX (n, n dibutylphthalate polystyrene xylene). Negative controls were performed for each sample by replacing the primary MAb with TBS to allow the assessment of non-specific binding of the secondary antibody. Sections of first trimester placental paraffin tissue collected from TOP patients served as positive controls. The average number of NDOG1 positive cells in two 20 μl aliquots of cell suspension from the 43 processed CV+ samples was determined under a normal light microscope (BH-2, Olympus) using a 20× objective lens. The number of syncytiotrophoblasts per ml of the original CV+ samples was then calculated. ANOVA using the LSD test for multiple comparisons was applied to log transformed data (mean ± SEM) and a p value < 0.05 was deemed significant.