Do extravillous trophoblasts isolated from maternal blood and cervical canal express the same markers?

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Research Article

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Abstract

Introduction Two approaches have been suggested for cell-based non-invasive prenatal testing (cbNIPT); A) enrichment of extravillous trophoblasts (EVTs) from maternal blood using antibodies targeting a combination of mesodermal and ectodermal markers and B) enrichment of EVTs from cervical swabs using antibodies targeting human leukocyte antigen G.

Objective To investigate whether a well-established protocol for enrichment of EVTs from maternal blood sample can be used on cervical swabs.

Methods A blood sample and a cervical swab from each of the ten women carrying male fetuses and undergoing first trimester termination of pregnancy were obtained. A previously published method, ‘trophoblast retrieval and isolation from the cervix’ was used for mucus dissolvement before enriching EVTs using antibodies against CD105 and CD141, as used for enrichment of EVTs from maternal blood. XY chromosome fluorescence in situ hybridization was performed to validate potential EVTs.

Results No EVTs (XY cells) were detected in the cervical samples. Only maternal cells of epithelial origin were identified. However, the number of EVTs in blood samples varied from 1-9 EVTs per 30 mL.

Conclusion A cbNIPT antibody-based protocol developed for enrichment of EVTs from maternal blood failed to enrich EVTs from the cervical canal of pregnant women in first trimester.

Introduction

Cell-based non-invasive prenatal testing (cbNIPT) may represent a superior alternative to cell-free fetal non-invasive prenatal testing (cffNIPT). The major argument in favor of cbNIPT is that the nucleated fetal cells contain the entire fetal genome, making it possible to perform a wider range of and more detailed genetic analyses [1–6]. Furthermore, unlike cffNIPT, cbNIPT is not significantly affected by increased maternal BMI [5, 7]. cbNIPT can be based on fetal leucocytes [8, 9] or nucleated red blood cells [10] found in maternal blood, but more promising and relatively well-established methods are based on extravillous trophoblasts (EVTS), circulating in maternal blood [1, 3, 4, 6, 11, 12],cell or retrieved from the cervical canal of pregnant women [13–17]. Interestingly, both methods are based on enrichment of EVTs but target different cell surface markers.

As regards to EVTs in maternal blood, ARCEDI Biotech ApS holds proprietary technology for immunomagnetic enrichment of EVTs using antibodies against the mesodermal cell surface markers CD105 and CD141, and staining of EVTs with a combination of fluorescent antibodies targeting ectodermal cytoskeletal markers [1, 3, 6]. In contrast, Trophoblast Retrieval and Isolation from the Cervix (TRIC) is based on EVTs retrieved from the cervical canal of pregnant women using cervical swabs and immunomagnetic enrichment targeting the EVT surface marker, human leukocyte antigen G (HLA-G) [13–17].
As illustrated in Fig. 1, trophoblasts originate from chorionic villi [18, 19]. Villous cytotrophoblasts proliferate in cell columns at the tip of the villi and invade the decidual stroma, where they start expressing HLA-G and mesodermal markers [18, 20]. These trophoblasts are called invasive interstitial EVTs, which may reach maternal circulation and potentially the cervical canal by different routes. HLA-G positive interstitial EVTs have been demonstrated in decidua basalis, uterine veins, arteries, and glands [13–17]. Two different routes have been proposed for EVTs reaching the cervical canal. At the margin of placenta, invasive interstitial EVTs may migrate directly into the uterine cavity or alternatively invade the uterine glands in decidua basalis and hereby reach the cervical canal during the first trimester [20]. Furthermore, invasive EVTs migrate from decidual stroma to the uterine veins [21] and spiral arteries displacing the vascular endothelium, and lining the vessel wall to establish feto-maternal circulation [19, 22]. This invasion is complex and involves transition from an epithelial to mesenchymal phenotype, which allows the EVTs to invade maternal vessels [23, 24] (Fig. 1).

It is unknown whether EVTs found in the cervical canal and maternal blood express similar cell surface markers. Hence, the aim of this study was to investigate, whether the antibodies used in a cbNIPT protocol enriching EVTs from maternal blood can enrich EVTs from maternal cervical swabs.

**Methods**

**Participants**

Inclusion criteria were nulliparous or parous pregnant women carrying male fetuses in gestational age (GA) 7 + 0 to 11 + 6 referred for surgical termination of pregnancy at Randers Regional Hospital, Denmark. Exclusion criteria were multiple gestations and active vaginal bleeding. All protocols, patient information and consent forms were approved by the Danish Research Ethics Committee (project ID: M-20110305).

**Blood processing**

Thirty mL of maternal blood was obtained in Cell-Free DNA BCT® tubes (Streck, USA) prior to termination of pregnancy. The maximum time from sample collection to sample processing was six hours. The gender of the fetus was determined by Y-chromosome specific real-time PCR from plasma and only samples from male fetuses were processed further. Gender PCR was performed using a protocol as previously described [1, 4, 6]. The blood samples underwent cbNIPT blood processing, which includes fixation with paraformaldehyde, followed by red blood cells lysis, washing with phosphate-buffered saline (PBS) (Gibco, pH 7.4 w/o Ca2 + and Mg2+) and magnetic-activated cell sorting (MACS) [1, 4]. Figure 2 illustrates the research method.

**Swab processing**

A cervical swab was obtained by a cytobrush, EndoCervex CytoBrush® (Rovers Medical Devices, The Netherlands), which was inserted 1.5-2.0 cm into the cervical canal and rotated 360 degrees clockwise. All samples were collected by the same researcher while women were under general anesthesia prior to
surgical termination of pregnancy. Nulliparous women were given vaginal misoprostol two hours before the procedure. The swabs were kept for maximum 6 hours at 4°C in 10 ml sterile PBS until processing. Dissolvement of cervical mucus was replicated in accordance with the TRIC protocol as described earlier [14–16]. This included adding 250 µl concentrated glacial acetic acid to the ice-cold PBS containing the cytobrush followed by 5 min incubation at room temperature. The sample was centrifuged at 4°C and the cell pellet washed with ice-cold PBS three times, followed by centrifugation and removal of supernatant [14–16]. Instead of using ethanol for fixation, we used paraformaldehyde-based fixation (shown in Fig. 2).

**Enrichment of extravillous trophoblasts**

Enrichment and staining of EVTs from blood samples and cervical swabs were performed identically by using Magnetic Activated Cell Sorting (MACS) (Miltenyi Biotech, Germany) in accordance with the cbNIPT protocol, as previously described [1, 4]. The cells were incubated with antibodies against CD105 and CD141 conjugated to magnetic beads before immunomagnetic enrichment on MACS columns, and stained with a cocktail of cytokeratin antibodies, before being smeared on FLEX IHC microscope slides (DAKO, USA). After air-drying, the glass slides were fixed in 2% paraformaldehyde, and mounted with Vectashield containing DAPI (4′,6-diamidino-2-phenylindole) (Vector Laboratories, USA). The slides with cell smears were scanned using a fluorescence microscope with an integrated scanner from MetaSystems. Potential EVTs were identified by their morphological characteristic, such as nuclear and cytokeratin staining.

XY chromosome fluorescence in situ hybridization (FISH) was performed to allow identification of cells with a Y chromosome, which indicated fetal origin (shown in Fig. 1).

**XY fluorescence in situ hybridization**

XY chromosome FISH was performed as previously described [25]. Briefly, chromosome specific repeat probes for CEP X alpha satellite (spectrum green, spectrum aqua) and CEP Y satellite III (spectrum orange, spectrum aqua) (Abbott Molecular, USA) were used for primary hybridization to the X and Y chromosome, respectively. Following hybridization, the slides from blood and cervix samples were scanned for Y chromosome signals indicating fetal origin (shown in Fig. 1). For final validation of EVTs, re-hybridization with reverse probe colors was performed using chromosome specific repeat probes. Positive controls of male cells from epithelial mouth mucosa were processed concurrently to confirm successful hybridization to the Y chromosome.

**Results**

Blood samples and cervical swabs were obtained from ten pregnant women carrying male fetuses (GA average 9 + 3, range GA 7 + 2 to 11 + 4. Replicating the TRIC protocol for mucus dissolvement and subsequently cbNIPT protocol for enrichment of EVTs we did not detect any XY cells in the cervical samples, thus no EVTs were identified by this method. We isolated EVTs from all blood samples (average 4.1, range 1 to 9). Figure 3A shows maternal cells with XX FISH signals enriched from a cervical swab.
due to unspecific binding in the enrichment process. Figure 3B shows a positive control smear with XY FISH signals. Figure 4 shows an EVT isolated from a maternal blood sample with fluorescent cytokeratin staining (A) and subsequent XY chromosome FISH validation (B). Among the EVTs identified in maternal blood by cytokeratin staining, 68% were confirmed by XY chromosome FISH. The remaining cells were either lost or gave no signals due to small and condensed nuclei.

**Discussion**

We were not able to enrich EVTs from cervical swabs by using a well-established cbNIPT protocol for enrichment of EVTs from maternal blood using CD105 and CD141. To our knowledge, this is the first study to test a cbNIPT protocol developed for blood samples on cervical swabs.

A strength of this research is that it is based on two well-established methods, TRIC and cbNIPT. The cervical swab enrichment and blood sample enrichment steps were performed identically and simultaneous in the laboratory and only the first step of swab processing and blood processing differed.

Our negative results may be explained by at least two hypotheses. First, the EVTs in cervical canal lack CD105 and CD141. It is likely that the endocervical EVTs and the circulating EVTs differ in antigen expression. The circulating EVTs may well be unique in their expression of both mesodermal and ectodermal markers, since they adopt to maternal circulation by expressing CD105 and CD141, allowing them to invade maternal vessels (Fig. 1) [1, 4]. Second reason for the lack of EVTs in the cervical swabs could be technical. Despite, adhering to the well-established TRIC protocol, we cannot exclude that we failed to reproduce the protocols and failed to recover fetal cells from the cervical canal due to simple technical reasons such as sample collection, sample medium or inadequate mucus dissolution.

Nevertheless, it is well established by others that EVTs can be retrieved with endocervical swabs in early pregnancy [13–17, 26–30]. One study based on isolation by cell size [29] enriched approximately ten EVTs pr. cervical swab [29], while more than 1000 EVTs pr. cervical swab were isolated with immunomagnetic enrichment with HLA-G (TRIC) [14]. However, even though recent advances have been described, no prenatal test based on cells isolated from cervical swabs has yet been developed.

In conclusion, CD105 and CD141 do not constitute an alternative to HLA-G when it comes to EVT isolation from the cervical canal of first trimester pregnant women. Future research should be directed towards the comparison of endovascular and endocervical EVTs, which can shed light into their unique protein expression pattern.

**Declarations**

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Competing interests

The author(s) declare no competing interests.

Statement of ethics

All protocols, patient information and consent forms were approved by the Danish Research Ethics Committee (project ID: M-20110305). This research was conducted ethically in accordance with the guidelines for human studies and in accordance with the World Medical Association Declaration of Helsinki.

Consent to participate

All participants have given their written informed consent prior to inclusion in the research study.

Availability of data and material (data transparency)

All data and material gathered can be made available upon request.

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Author Contributions

CK conducted the study. KR, LH, RS and IV supervised laboratory work and helped finalizing the manuscript. PB assisted in sample collection and finalizing the manuscript. NU initiated and supervised the study. CK wrote the manuscript in collaboration with NU and RS. All authors read and approved the final manuscript.

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Figures
Figure 1

**EVT invasion routes**

Illustration of the pregnant uterus and developing placenta in first trimester of pregnancy and EVT invasion routes (zoom box, right). During implantation placenta contacts the maternal decidua to form feto-maternal interface. Villous trophoblasts (green cells) partly lose their epithelial phenotype as extravillous trophoblasts (EVTs) and start expressing different surface markers such as HLA-G (yellow cells). HLA-G positive EVTs have been demonstrated in decidua, uterine veins, uterine glands, and spiral arteries. NB: the figure is only schematic. Uterine veins and glands are not demonstrated.
Figure 2

Diagram showing the research method design

The processing steps of blood samples and cervical swabs were identical except from the first step allowing release of EVTs by red blood cells lysis, and mucus dissolvement, respectively.
Blood processing and swab processing were followed by incubation with antibodies against CD105 and CD141 conjugated with magnetic beads, and subsequent magnetic activated cells sorting and staining with fluorescent cytokeratin antibodies. Slides containing the enriched cell population were scanned in a fluorescence microscope and potential EVTs were identified. As a final step, fluorescence in situ hybridization (FISH) was performed and potential EVTs manually validated for Y chromosome signals before being scanned for cells containing Y chromosome signals representing EVTs.

Figure 3

*Cervical swab, enriched (A) and positive control (B)*

Maternal cervical cells of epithelial origin in immunofluorescence microscope after fluorescence in situ hybridization for X chromosome (green) and Y chromosome (red) (a). Positive control from male mouth mucosa after fluorescence in situ hybridization (FISH) for X chromosome (green) and Y chromosome (red) (b)
Figure 4

*Maternal blood, enriched*

Identification of an EVT from a blood sample by immuno-fluorescence microscopy (patient 3, GA 10+6). EVT stained with antibodies against selected cytokeratins (green) and DAPI nuclear stain (blue) (a). The same cell shown after validation with fluorescence in situ hybridization (FISH) for X chromosome (green) and Y chromosome (red) to confirm the fetal origin (b).