ORIGINAL ARTICLE

Identification of embryo–fetal cells in celomic fluid using morphological and short-tandem repeats analysis

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ABSTRACT

Objective The main problem to wide acceptability of celocentesis as earlier prenatal diagnosis is contamination of the sample by maternal cells. The objective of this study was to investigate the cellular composition of celomic fluid for morphological discrimination between maternal and embryo–fetal cells.

Method Celomic fluids were aspired by ultrasound-guided transcervical celocentesis at 7–9 weeks' gestation from singleton pregnancies before surgical termination for psychological reasons. DNA extracted from celomic fluid cells showed the same morphology, and quantitative fluorescent polymerase chain reaction (PCR) assay was performed to evaluate their fetal or maternal origin.

Results Six different types of non-hematological maternal and four different types of embryo–fetal cells were detected. The most common maternal cells were of epithelial origin. The majority of embryo–fetal cells were roundish with a nucleus located in an eccentric position near the wall. These cells were considered to be erythroblasts, probably derived from the yolk sac that serves as the initial site of erythropoiesis.

Conclusions The combined use of morphology and DNA analysis makes it possible to select and isolate embryo–fetal cells, even when maternal contamination is high. This development provides the opportunity for the use of celocentesis for early prenatal diagnosis of genetic diseases and application of array comparative genomic hybridization. © 2016 John Wiley & Sons, Ltd.

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INTRODUCTION

The celomic cavity (CC) is the largest space inside the gestational sac during the first 9 weeks' gestation; it reaches its maximum volume at 7–9 weeks and disappears at around 12–13 weeks.[1] The composition of celomic fluid (CF) is an ultrafiltrate of maternal serum with the addition of specific placental and secondary yolk sac proteins. Consequently, the CC can be considered as a physiologic liquid extension of the early placenta, acting as possible reservoir for nutrients needed for the developing embryo.[2–4] Chorionic villus sampling (CVS) and amniocentesis are the invasive conventional prenatal diagnostic procedures for monogenic diseases or karyotype analysis. Use of CVS is restricted from 11 weeks onwards because when it is carried out earlier there is increased risk of miscarriage and development of limb defects.[5–7] Similarly, amniocentesis is restricted to 15 weeks onwards because when it is carried out earlier there is increased risk of miscarriage and development of talipes.[8–10] Recent advances have allowed the widespread introduction of noninvasive prenatal testing (NIPT) using cell-free DNA (cffDNA) in maternal blood. The only physical risks associated with the procedure are those normally because of the blood draw. However, although cffDNA can be detected in maternal plasma as early as 5–7 weeks, test results are more accurate after 10 weeks because of an increase in amount of cffDNA increases over time.[11,12] Furthermore, for prenatal diagnosis of single-gene disorders, only the paternal mutations can be diagnosed. Therefore, this technique could only reduce the rate

of invasive testing by 50% when the parents have different mutations.

Celocentesis can successfully provide a sample in nearly all cases from as early as the 7th week of gestation.[13–17] DNA extracted from less than 1 mL of CF can be analyzed using polymerase chain reaction (PCR) and other techniques, and the method could be used for molecular karyotyping and prenatal diagnosis of genetic conditions, including thalassemia, cystic fibrosis and spinal muscular atrophy.[18–20] However, the introduction of celocentesis in prenatal diagnosis has been hindered by a high grade of maternal cell contamination (MCC). The possibility of having information on the accurate cellular composition of CF and of isolating fetal cells could make celocentesis a reliable early prenatal diagnosis procedure.[21–24]

The objective of this study was to investigate the cellular composition of CF for morphological discrimination between maternal and embryo–fetal cells.

MATERIAL AND METHODS

Sampling

CFs were collected by ultrasound-guided transcervical celocentesis at 6^{+6}–9^{+2} weeks' gestation from 122 singleton pregnancies before surgical termination for psychological reasons. All women provided written informed consent to participate in the study which was approved by the institutional Review Board (No 80, 26 January 2005). After general anesthesia was performed, the external genitalia and the vagina were carefully cleansed with an antiseptic solution, and transvaginal sonography with a 5-MHZ transducer was performed for the measurement of the fetal crown–rump length and fetal heart rate, identification of the amniotic membrane, celomic space and yolk sac and diagnosis of any uterine abnormalities. A needle was then introduced into the CC, and CF was successfully aspirated in two syringes. For the first, 0.5 mL was discarded because it contained maternal traces of blood, and for the second 2–5 mL was used for analysis. Subsequently, suction termination was carried out, and placental tissue and maternal venous blood were collected for molecular tests.

Cell pick-up

Samples were centrifuged at 1000 rpm for 7 min, supernatants were aspirated and 100 μL of 0.9% NaCl was then added to each sample and placed in a petri dish. Optical phase contrast microscopy (40× magnification) was used to identify cells showing the same morphology which were then aspirated one by one using a micromanipulator with 45-μm glass micropipet and placed in a drop of 0.9% NaCl in the same petri dish. Ten different cell types were isolated based on the morphological criteria and were further subdivided into 159 pools containing 5–20 cells with the same morphology (Figure 1). Thirty-two pools for type 1 cells, six for type 2, 44 for type 3, seven for type 4, 38 for type 5, eight for types 6 and 8, seven for type 7, four for type 9 and five for type 10 were analyzed (Table 1).

DNA extraction

Each pool of cells was aspirated from the petri dish and placed into a 0.2-mL Eppendorf tube containing 4 μL of 0.05 M NaOH and 0.5 μL of 1% triton. Tubes were then placed in a thermal cycler, incubated at 45 °C for 25′ and at 94 °C for 25′ and then 1 μL of 1 M TrisHCl pH 8 was added. DNA from placental samples and maternal blood were digested by Proteinase K incubation and extracted by salting-out procedure.[25]

Cell origin association

A sensitive multiplex fluorescence PCR (QF-PCR) was used to evaluate the origin of cells. Short tandem repeat (STR) of highly variable chromosomal markers located on chromosome 13 (D13S325, D13S631, D13S634, D13S305), chromosome 18 (D18S535, D18S386, D18S499, D18S391), chromosome 21 (D21S1435, D21S1411, D21S1442, D21S1414), and chromosomes X and Y (AMXY, HPRT, SRY, DXS8377, DXS1187, DXS6803, DXS6809) were analyzed. Fifteen microliters of 2× Platinum Multiplex PCR Master Mix (Life Technologies, Carlsbad, CA, USA), 0.3 μL of a mix contained 3–10 pmol of each primer and 11.5 μL of distilled water were added in the tubes containing lysed cells. DNA (20 ng) from placenta and maternal venous blood were amplified in a final volume of 20 μL. After one cycle of denaturation at 95 °C for 2′, PCR was performed for 35 repeating cycles at 95 °C 30″,

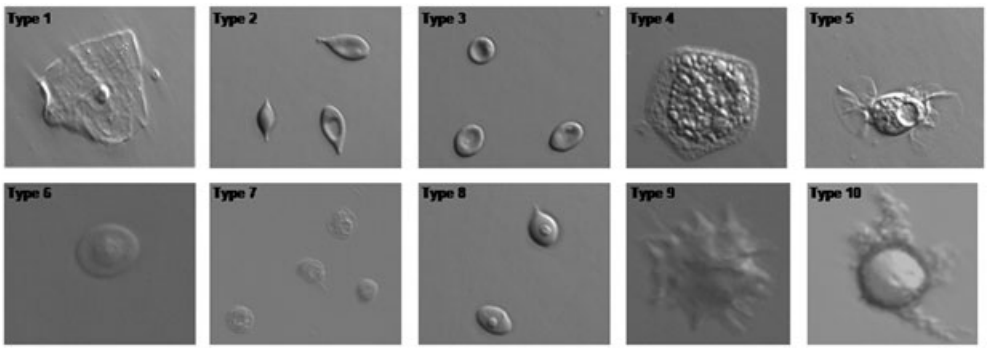

Figure 1 Morphology of celomic fluid cells observed by optical phase contrast microscopy. The features are described in Table 1

Table 1 Morphologic features of celomic fluid cells observed by optical phase contrast microscopy. The table reported the morphology of each cell, the percentage average found in the CF, the origin and the number of pools examined

Cell type	Diameter (μm)	Morphology	Proportion in the sample (%)	Origin	Pool cells examined
1	30	Flattened cell with a small nucleus like epithelial cell of vaginal origin	20	Maternal	32
2	16	Elongated cell with an evident nucleus; this could be a fibroblast	5	Embyo-fetal	6
3	9–14	Round cell with picnotic eccentric nucleus; this could be an erythroid precursor	5–30	Embyo-fetal	44
4	40	Nucleated big ovoidal cell with rough surface	15	Maternal	7
5	15–18	Round cell with rough surface and pseudopodia with no visible nucleus; this could be of neuronal origin	5–30	Embyo-fetal	38
6	5–8	Round cell with flattened nucleus	Rare	Maternal	8
7	6–15	Flattened cell with wrinkled surface without an evident nucleus.	Rare	Maternal	7
8	6–8	Elogated cell with evident nucleus on its surface; this could be an erythroid precursor	5	Embyo-fetal	8
9	8–12	Small cell with a surface similar to a star without a visible nucleus.	Rare	Maternal	4
10	8–12	Round cell with a thick dark color membrane and aspecific material attached on its surface. Nucleus is not visible.	Rare	Maternal	5

60 °C 90″, 72 °C 60″ and a final cycle at 72 °C for 30′. Thermal cycling was performed using the Gene Ampl 9700 (Applied Biosystems Foster City, CA, USA). Typing of PCR products was carried out on an ABI PRISM 3130 DNA, and the data were analyzed by GeneScan software analyzer (Applied Biosystems Foster City, CA, USA). DNA data from CF, placenta and maternal blood were compared.

RESULTS

On inspection, CFs had a yellow color, with varying intensity in different samples. Five CFs were red colored because of the presence of blood traces and in four CFs there were traces of mucus which were removed with a pipet. A large variability of cell density and a wide variety of cells with different size and morphology were observed by optical phase contrast microscopy at 40× magnification. These cells included red blood cells, trophoblast cells, lymphocytes, erythroblasts, mesenchymal, fibroblast and many others of unknown origin. Figure 2 represents an example of the electropherograms of QF-PCR polymorphic STR markers for samples of maternal or fetal origin. Figure 2-C shows in particular the result of STR D13S631obtained from type 1 cell's DNA, while Figure 2-D shows the result of STR D13S631obtained from type 3 cell's DNA. Sequences of fluorescent peaks of maternal cell's DNA are represented by the presence of both maternal alleles (C), while if one maternal and one paternal peaks were presented, pooled cells were attributed to fetal origin (D).

A total of 63/159 pools were of maternal origin and 96/159 were of fetal origin. Four types of fetal cells (types 2, 3, 5, 8) and six of maternal origin (types 1, 4, 6, 7, 9, 10) were with certainty identified (Figure 1).

Maternal type 1 cells were observed in more than 70% of CFs sampled. These cells are big with a size of more than 30 μm showing morphological aspects of epithelial cells and are characterized by a flattened structure with a hardly visible nucleus (Figure 1). Type 2 cells were of fetal origin. They are elongated ovoid with a small, noticeable nucleus attached on the inner side of the cell membrane (Figure 1). These cells are present in low frequency in the CF samples observed. Type 3 cells were the most frequently observed fetal cells in all CFs examined. They are characterized by a round form of variable size between 9 and 14 m with the presence of an evident nucleus placed in an eccentric position near the cell membrane (Figure 1). Type 4 maternal cells were the largest ones observed, that is, more than 40 μm. The surface of these cells is characterized by the presence of a rough, irregular surface (Figure 1). These cells were rare. Type 5 has a round form with a size of about 15–18 m. Their surface is rough without an evident nucleus but characterized by filamentous excrescence as pseudopodia in the surface membrane (Figure 1). These cells are of fetal origin and were observed frequently in the CFs examined. Type 6 maternal cells were rarely observed in the CFs. They appeared roundish with a large and evident and homogeneous nucleus in the middle of the cell (Figure 1). These are maternal cells. Type 7 maternal cells are rounded without any evident nucleus (Figure 1). These cells were rarely observed. Type 8 cells are of fetal origin. They are round or ovoid with and evident nucleus at the center of the cell. These cells were rarely present (Figure 1). Types 9 and 10 are both of maternal origin. Their sizes were similar (8–12 mm) and were rarely present. Type 9 cells presented a fringed surface without an evident nucleus. Type 10 is rounded with a clear central portion and the presence of apparently mucous material bound on the surface (Figure 1).

About 30% of CFs observed (48/122) appeared very rich in fetal cells (more than 4–6 hundreds) while other CFs contained only few dozen of cells (10–30 cells). No uniformity of cell types was observed across the different CF or in function of gestation age.

DISCUSSION

Morphological and DNA studies have demonstrated that the CF contains a wide variety of cells of maternal and embryo–

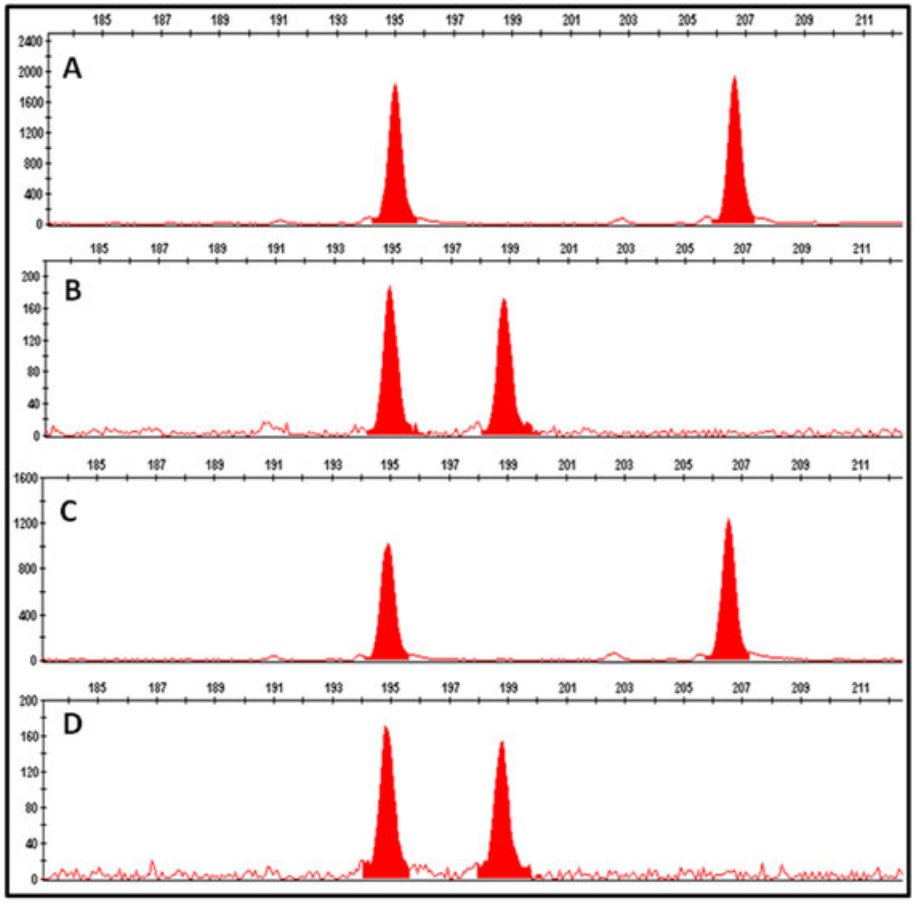

Figure 2 Electropherograms of QF-PCR products from maternal blood (A), placental tissue (B) and two types of celomic fluid cells, one of maternal (C) and the other of fetal origin (D).The pure fetal origin of the pooled cells was confirmed by the different STR profiles observed in the mother. Particularly C shows the result of STR D13S631obtained from type 1 cell's DNA, while D shows the result of STR D13S631obtained from type 3 cell's DNA

fetal origin. Our study shows that the CF contains at least six different types of maternal cells. Their source is likely to be because of contamination from tissues crossed during the sampling; however, their possible origin from maternal–fetal celomic circulation cannot be excluded. Four types of embryo–fetal cells have been identified: one type could be fibroblast cells, another with pseudopodia could be neuronal cell precursors and two others are megaloblasts (CD 71+/CD 45−) previously reported.[26,27]

Hematopoiesis in the embryo starts in the 2nd or 3rd week of gestation when yolk sac blood islands develop clones of primitive nucleated erythroblasts or megaloblasts.[28–30] Detailed analysis of these embryonic cells revealed a prevalent megaloblast pattern with CD71+/CD45− membrane expression, consistent with embryonic erythroid precursors.[31] The presence of such precursors in CF may suggest cellular trafficking between the embryo and the CC. The possibility of early access to the CC in combination with cell trafficking between embryonal compartments could open a novel strategy for *in utero* cell transplantation.[32–34] Megaloblasts and cells with pseudopodia are the main cellular components used in our center for prenatal diagnosis by celocentesis because they are frequently present and easily identifiable because of their morphology. The robustness and reproducibility of this methodology to classify these cells in relation to their morphology were demonstrated by the correct association of their morphology to their origin in all pooled cells analyzed. As recently reported,[27] 302 CF were examined for prenatal diagnosis of hemoglobinopathies by applying a flow work which required microscopic assessment and cell selection by micromanipulation of fetal cells based on their morphology. The results obtained by celocentesis in this clinical diagnostic trial were all subsequently confirmed with amniocentesis or CVS. Three main problems have so far limited the use of celocentesis as an early prenatal diagnostic procedure alternative to CVS and amniocentesis. The first was the low amount of fetal DNA recovered; this problem has now been overcome with the use of methodologies such as nested PCR, which can adapt to a small amount of DNA. The second problem was the MCC. The present study has demonstrated the feasibility to distinguish between embryo–fetal and maternal cells in CF. It is now possible to isolate embryo–fetal cells after positive selection using antibodies or individually by micromanipulator picking-up making it possible to solve concerns about maternal DNA contamination.[27] The third problem relates to uncertainty of the safety of the technique. We have carried out this procedure in a large number of continuing pregnancies and

demonstrated that the associated risk of miscarriage is very low (data not published yet). The main limitation of our study is lack of complete characterization of maternal and embryo–fetal cells, because of the small amount of samples. We are currently conducting cell cultures, fluorescence and gene expression studies. However, single-cell genome sequencing and single-cell transcriptomics have recently emerged as powerful tools to study the biology of single cells at a genome-wide scale. These new methods are most efficient for studies of genotype–phenotype association within single cells to definite cell types and states. RNA analyses further permit to generate more information for genetic heterogeneity and cell-lineage relationships in single cell derived tissue[35–37]

CONCLUSION

This study has demonstrated that (i) CF contains a wide variety of embryo–fetal and maternal cells without uniformity of cell types across the different patients assessed or as a function of gestational age; (ii) the number of embryo–fetal cells is highly variable; and (iii) selection and isolation of embryo–fetal cells from CF is feasible. The combined methodology of morphological assessment and STR analysis make it possible to select and isolate embryo–fetal cells, even when maternal contamination is high. The diagnostic procedure for contaminated CF samples requires selection of pure fetal cells of type 2/3/5 and 8 to remove contamination and makes celocentesis a reliable procedure for early prenatal diagnosis of Mendelian disorders and chromosomal analysis by standard[38] methodology or microarray comparative genomic hybridization (CGH).[39,40] Our experimental results, described in this manuscript, and the 353 prenatal diagnosis performed by celocentesis for hemoglobinopaties,[27] without any diagnostic errors, allow us to propose the procedure of the fetal cell selection to solve the contamination in the CF samples. Celocentesis remains an attractive technique for early prenatal diagnosis of genetic disorders as demonstrated by a growing demand from couples at risk for hemoglobinopathies coming from all regions of Italy.

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Authorship

All authors have contributed to the study design, revised and approved the manuscript. In addition, A.G, K.H.N, G.M. and A.M. drafted and approved the manuscript; F.L., G.S., A.V., A. A. and C.P. performed experiments using the selected cells, G.D., C.J., G.M. and F.P. G.P. collected samples of CF by celocentesis and V.C. and G.S. were involved in patient enrollment.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- The risk of contaminated celomic fluid by maternal cells may preclude molecular analysis for genetic test. The biochemical characteristic of celomic fluid was extensively studied, but knowledge about the presence of cellular elements and their origins in this compartment are still limited. Only the presence of functional embryonic erythroid precursors, which are megaloblasts, in the celomic cavity was already demonstrated, but the complete cell composition of this fluid remains unknown.

WHAT DOES THIS STUDY ADD?

- The cell composition of the celomic fluid contains 10 different cell types of which six are of maternal origin and four are types of embryo–fetal cells. These cells can be selectively distinguished by morphological criteria and selectively isolated.

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