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Feasibility of DNA diagnosis of haemoglobinopathies on coelocentesis

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Received 30 November 2010; accepted for publication 20 January 2011
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Summary

At 5–12 weeks of gestation the amniotic sac is surrounded by celomic fluid, which contains cells of fetal origin. This fluid can be sampled by celocentesis, which involves the ultrasound-guided insertion of a needle through the vagina. The aim of this study was to examine the feasibility of prenatal diagnosis of haemoglobinopathies from the celomic fluid using a specific protocol. Celocentesis was performed at 7–9 weeks gestation in 26 singleton pregnancies at risk for haemoglobinopathies. In 25 cases more than 30 fetal cells were recovered from the celomic fluid and in all these cases molecular analysis for haemoglobinopathies was possible and the results were confirmed by subsequent chorionic villus sampling or amniocentesis. The results of this study suggest that reliable diagnosis of thalassemia syndromes can be performed from 7 weeks gestation by celocentesis. Further work is necessary to demonstrate the safety of celocentesis before widespread use.

Keywords: haemoglobinopathies, thalassemia, prenatal diagnosis, celocentesis, celomic fluid.

Haemoglobinopathies are a major public health problem worldwide (Weatherall, 2010). ß-thalassemia and haemoglobin disorders account for 3·4% of deaths in children <5 years of age (Modell & Darlison, 2008). Prevention of thalassemia major can be achieved by population screening in combination with prenatal diagnosis (Rosatelli *et al*, 1986; Harteveld *et al*, 2009).

Prenatal diagnosis of chromosomal disorders and genetic abnormalities necessitates the analysis of fetal material obtained by chorionic villus sampling (CVS) at 11–13 weeks gestation or amniocentesis from 16 weeks onwards. An alternative technique for earlier diagnosis is celocentesis (Jurkovic *et al*, 1993; Makrydimas *et al*, 1997, 2002, 2004). At 5–12 weeks gestation the amniotic sac is surrounded by celomic fluid (CF), which contains cells of fetal origin (Jurkovic *et al*, 1993; Renda *et al*, 2010). This fluid can be sampled by a technique that involves the ultrasound-guided insertion of a needle through the vagina from as early as 6 weeks. The desirability of such technique is not only based on the feasibility of achieving prenatal diagnosis several weeks earlier than with CVS but also on the potential for in-utero stem cell transplantation before the fetus becomes immunologically competent. However, studies investigating the feasibility of prenatal diagnosis from fetal DNA extracted from cells in the CF reported failure rates of more than 40%, which was attributed to the small number of celomic cells, the high degree of maternal contamination and the unknown nature of the maternal and fetal cells in CF (Jouannic *et al*, 2006, 2008).

In this study we present a CF-specific protocol that maximizes the success rate of prenatal genetic testing using this fluid.

Materials and methods

The institutional review board approved the study, which complied with the declaration of Helsinki and the participating couples provided written informed consent (Hospital Ethical Committee authorization on date 26 January 2005, No 80). The patients included in this study are part of an ongoing investigation examining the safety of celocentesis.

doi:10.1111/j.1365-2141.2011.08621.x

Sampling

Celocentesis was carried out at 7–9 weeks gestation in 26 singleton uncomplicated pregnancies at risk for haemoglobinopathies, including 15 for β-thalassemia, nine for Sickle-Cell/beta-thalassemia and two for β-thalassemia and Hb Lepore Boston (Table I). The procedure was offered to all women seeking prenatal diagnosis of haemoglobinopathy at our centre. The procedure, performed by the same operator (George Makrydimas), essentially involved transvaginal sonography and the insertion of a 20 G needle through the fornix into the celomic cavity (Fig 1; Makrydimas *et al*, 2002). Samples of CF were aspirated into three different syringes (0·2, 0·2 and 0·6 ml). The first two samples were discarded to reduce the risk of contamination by maternal blood and the third sample was used for analysis.

Microscopical observation

An aliquot of CF (50 μl) was put in a petri disk and observed by optical microscopy to assess the number and composition of cells as previously reported (Renda *et al*, 2010).

Purification

The CF samples in which the percentage of maternal cells were more than 5% of the total cell content were purified using anti-CD45 and anti-CD105 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for negative selection. The CF sample was placed in an Eppendorf tube and centrifuged at 400 *g* for 10 min. The pellet was then dissolved in 50 μl of phosphate-buffered saline (PBS) and 2·5 μl each of anti-CD 45 anti-CD 105 MicroBeads (Miltenyi Biotec) were added and prior to incubation at 4°C for 15 min. Subsequently, PBS (500 μl) was added to the tube and centrifuged at 400 *g* for 10 min and the pellet re-dissolved in PBS (550 μl). The sample was then applied to a MiniMACS separation column (Miltenyi Biotec) and all the liquid containing fetal cells (negative selection) was eluted into a clean Eppendorf tube. The pellet of cells was collected by centrifugation at 13400 *g* for 10 min, dissolved in 50 μl of 5% IstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) and incubated at 56°C for 30 min and at 95°C for 10 min. The supernatant (45 μl) containing DNA was removed and transferred to a Eppendorf tube (0·5 ml volume).

Table I. Description of clinical and laboratory findings in samples obtained by celocentesis.

Case	Parental mutation	Gestation (weeks)	Sample (μl)	Total fetal cells	Maternal cells contamination before purification (%)	Fetal genotype
1	IVS II-745/β^S	8 + 5	600	380	25	IVS II-745/β^S
2	Codon 39/Codon 39	8 + 6	630	530	10	Codon 39/Normal
3	IVS I-110/β^S	8 + 5	550	610	20	Normal/Normal
4	IVS I-110/Codon 39	7 + 1	540	370	65	Codon 39/Normal
5	IVS II-745/IVS-I-6	8 + 2	590	410	15	Normal/Normal
6	β^S/Codon 6 (-A)	7 + 2	610	720	5	β^S/Codon 6 (-A)
7	IVS I-110/IVS I-110	7 + 1	560	130	60	IVS I-110/IVS I-110
8	β^S/β^S	8 + 6	480	210	40	Normal/Normal
9	β^S/IVS I-110	8 + 3	450	500	15	β^S/IVS I-110
10	β^S/Codon 39	8 + 4	650	250	20	Codon 39/Normal
11	IVS I-1/β^S	8 + 6	650	700	10	IVS I-1/Normal
12	Codon 39/IVS I-110	8 + 5	500	300	15	Codon 39/IVS I-110
13	Codon 39/IVS I-110	8 + 2	600	480	15	IVS I-110/Normal
14	Hb Lepore/Codon 39	8 + 1	600	520	5	Hb Lepore/Normal
15	Codon 39/IVS II-1	8 + 0	520	4000	5	Normal/Normal
16	Codon 39/IVS II-745	8 + 4	500	2000	5	Codon 39/Normal
17	β^S/IVS I-6	9 + 2	670	160	40	β^S/IVS I-6
18	-101/IVS II-745	8 + 3	710	6400	5	Normal/Normal
19	IVS I-110/Hb Lepore	8 + 4	640	180	25	Normal/Normal
20	IVS I-6/IVS I-110	8 + 4	620	10	///	CF inadequate for diagnosis
21	IVS I-110/Codon 39	7 + 4	600	4800	5	IVS I-110/Codon 39
22	IVS I-110/Codon 39	8 + 5	680	120	25	IVS I-110/Normal
23	IVS II-745/Cod 39	8 + 5	700	3600	5	IVS II-745/Codon 39
24	IVS I-110/Codon 39	8 + 4	650	140	30	IVS I-110/Codon 39
25	IVS I-6/IVS I-110	8 + 3	540	200	30	IVS I-6/Normal
26	IVS I-6/β^S	7 + 4	610	190	30	IVS I-6/Normal

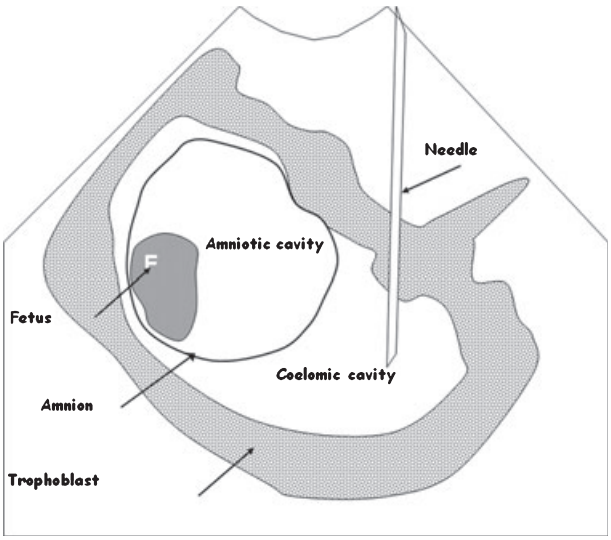

Fig 1. Schematic representation of celocentesis.

Molecular analysis of HBB (β-globin gene)

To analyse *HBB* (MIM#141900) levels, polymerase chain reactions (PCRs) were performed in 30 μl reactions using 5 μl of extracted DNA, 200 μmol/l of each dNTP (Roche Diagnostics GmmH, Mannheim, Germany), 1 × PCR buffer (Invitrogen, Valley Blvd, San Diego, CA, USA), 1·5 mmol/l $MgCl_2$, 10 μmol/l of each primer and 1·5 units of Taq Polymerase (Invitrogen, San Giuliano Milanese, Milano, Italy). The reaction mix was denatured at 96°C for 5 min. The reaction was performed for 35 cycles of 94°C 30 s, 55°C 30 s and 72°C for 45 s followed by 10 min at 72°C. Specific primers were used to amplify one or two fragments of *HBB* from −130 nucleotides 5′ to the CAP site to +150 nucleotides 3′ to the Poly-A. Amplified fragments were successively analysed by the direct sequencing method using BigDye Terminator 3.1 Cycle Sequencing kit (Applied BioSystems, Foster City, CA, USA) and run on ABI PRISM 3130 DNA Analyser (Applied BioSystems). *HBB* polymorphisms (Cd 2 C → T, IVSII nt 16 C → G, IVS II nt 26 T → G, IVS II nt 74 G → T, IVSII nt 81C → T nd IVSII nt 666 T → C) presented in both fragments were also analysed to check contamination with maternal DNA and for linkage analysis.

Maternal contamination control

Maternal contamination of the CF sample was evaluated before and after negative selection of maternal cells using a very sensitive multiplex fluorescence PCR (QF-PCR), containing primers for highly variable microsatellites of many chromosomal markers (STR) 13, 18, 21, X and Y (D13S631, D13S634, D13S258, D18S535, D18S386, D21S1435, D21S1411, D21S1414, AMXY, X22, HPRT, DXS6803, DXS6809). Products were denatured and fragments of different sizes were run on ABI PRISM 3130 DNA Analyser (Applied BioSystems). Data were analysed by GeneScan software (Applied BioSystems) (Fig 2).

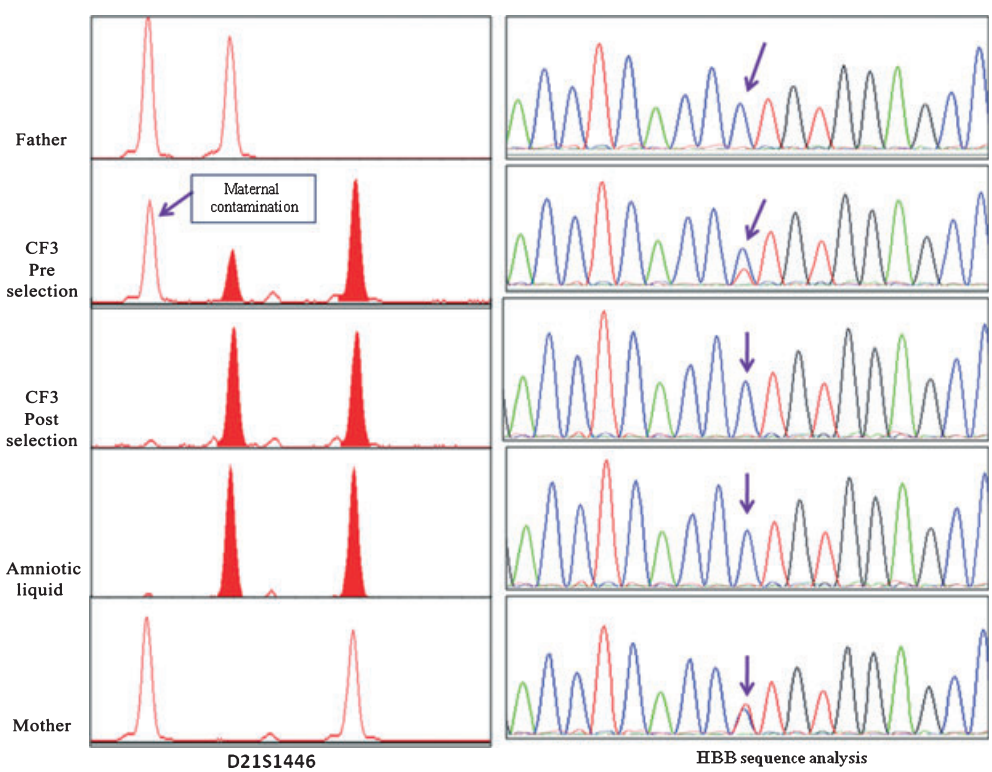

Fig 2. DNA fingerprinting by QF-PCR and mutation detection by sequencing analysis of coelomic fluid before and after purification are shown.

Results

Sampling

Aspiration of CF was possible in all cases and was not associated with a change in fetal heart rate. The volume of the CF sample used for diagnosis was 450–710 μl (Table I). On the basis of previous investigations (data not shown), only samples containing more than 30 fetal cells were considered adequate for molecular analysis and this was achieved in 25 of our 26 cases. One sample contained only 10 cells was considered inadequate for further analysis.

Microscopical observation

The presence of megaloblastic and other cells was observed, as previously reported (Fig 3; Renda *et al*, 2010). Surface antigens were characterized as CD71+ (fetal cells), CD45+ (maternal white cells) and CD105+ (mesenchymal/endothelial cells). The total number of fetal cells, estimated manually in a Burker chamber, was 10-6400 (Table I).

Purification

The CF samples were considered appropriate for molecular prenatal *HBB* without preventive purification in seven cases. The other 18 CF showed reduction of contamination to <1%, after negative selection of maternal cells.

Molecular analysis

This was conducted in 25 of the 26 samples. PCR amplification of DNA was proportional to the number of observed embryo-fetal erythroid cells (Renda *et al*, 2010). In nine cases the fetus was affected by thalassemia major and sickle-cell/beta-thalassemia and the parents decided to terminate the pregnancies. Ten and six were carriers and normal, respectively. Molecular analysis of prenatal diagnosis was confirmed by CVS or amniocentesis at 12 or 16 weeks gestation. Moreover, molecular diagnosis was also confirmed on placental tissue following pregnancy termination in those cases with affected fetuses.

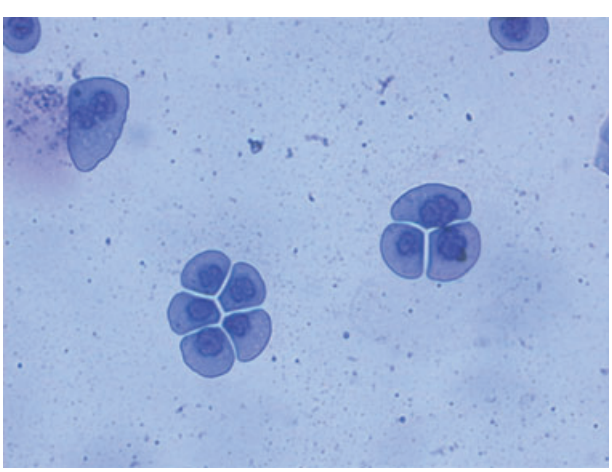

Fig 3. May Grunwald-Giemsa staining showing erythroid megaloblasts in coelomic fluid. Original magnification ×40.

Discussion

This study has established a specific protocol for the assessment and analysis of CF samples that has made it possible to achieve accurate prenatal diagnosis during the embryonic stage of intrauterine life.

The critical components of the protocol were firstly, expertise in obtaining CF samples and discarding the first samples with high maternal contamination, secondly, microscopical examination of the CF sample immediately after celocentesis to estimate the total number of fetal cells, and thirdly, purification of the sample to minimize the number of maternal cells. The findings of the study confirm our previous results that CF contains embryo-fetal erythroid precursors, megaloblasts (CD 71+/CD 45−) and maternal white cells (CD 45+) (Renda *et al*, 2010). All CF samples showing maternal contamination exceeding 5% were purified successfully using anti-CD45 and anti-CD105 for negative selection of maternal white blood cells and endothelial mecenchymal cells. Accurate prenatal diagnosis of haemoglobinopathies was achieved in all CF samples containing more than 30 fetal cells. In all 25 cases with an adequate number of fetal cells, the diagnosis was correct and confirmed by CVS, amniocentesis or placental tissue. The low success rate in diagnosis reported in previous studies is the inevitable consequence of failure to firstly, identify and distinguish between foetal and maternal cells and secondly, develop an effective method for removal of the maternal cells (Jouannic *et al*, 2006, 2008).

The CF contains embryo-fetal erythroid precursors or megaloblasts (Migliaccio *et al*, 1986; Renda *et al*, 2010). Haematopoiesis 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 (Valtieri *et al*, 1989; Stamatoyannopoulos, 2005; Zambidis *et al*, 2005). Detailed analysis of these embryonic cells revealed a prevalent megaloblast pattern with CD71+/CD45− membrane expression, consistent with embryonic erythroid precursors (Migliaccio *et al*, 1986). The presence of such precursors in CF may infer that there is cellular trafficking between the embryo and the celomic cavity. In theory, the possibility of early access to the celomic cavity in combination with the cell trafficking between the embryonal compartments could also allow a novel strategy for *in utero* cell transplantation (Noia *et al*, 2004).

This study has shown that reliable molecular diagnosis is possible from CF provided the sample contains at least 30 fetal cells. Future studies will determine the feasibility of prenatal

diagnosis in CF samples containing less fetal cells by employing specific selection of such cells directly under the microscope and the application of techniques similar to those used in pre-implantation diagnosis. Ultimately, widespread clinical application of celocentesis will need to await the results of ongoing studies on the safety of this technique.

Acknowledgements

This work was possible thank to a grant supported by Foundation Franco and Piera Cutino (Palermo, Italy). The authors thank The Fetal Medicine Foundation (London, UK) for its collaboration.

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