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Distribution of fetal and embryonic hemoglobins in fetal erythroblasts enriched from maternal blood

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Background and Objectives. To determine the distribution of embryonic and fetal hemoglobin chains in fetal erythroblasts isolated from maternal blood in the first trimester of pregnancy and establish the feasibility of using these chains as markers for fetal cell identification.

Design and Methods. Maternal blood was obtained from 187 singleton pregnancies at 11-14 weeks of gestation immediately before fetal karyotyping by chorionic villus sampling. In all cases included in this study the fetal karyotype was normal. Fetal erythroblasts were isolated using triple density gradient separation and anti-CD71 magnetic cell sorting techniques. The enriched erythroblasts were stained with Kleihauer-Giemsa and with fluorescent antibodies for the zeta (ζ), epsilon (ε) and gamma (γ) globin chains. The percentage of fetal cells positive for each stain was calculated. Fluorescent *in situ* hybridization (FISH) for X and Y chromosomes was also performed. Comparison was made with the percentage of cells with positive Y-signal FISH in pregnancies with male fetuses.

Results. The percentage of fetal erythroblasts stained positive was 37% for the ζ and 95% for both ε and γ globin chains, as well as the Kleihauer-Giemsa staining. There was a significant association between the Kleihauer-Giemsa stained cells and those stained with ε and γ globin chains. There was also an association between cells with Y-signals and those stained with ε and γ globin chains.

Interpretation and Conclusions. Embryonic hemoglobin chains can be detected in the enriched fetal erythroblasts, with higher percentages of the ε rather than the ζ globin chains. These chains are therefore potentially unique markers to be used in the identification of cells of fetal origin from maternal blood for prenatal diagnosis of genetic and chromosomal abnormalities.
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Key words: fetal cells in maternal circulation, embryonic (ζ,ε) and fetal (γ) globins, specific marker, prenatal diagnosis

Isolation of fetal cells from the maternal circulation is a promising method for the prenatal diagnosis of chromosomal and genetic disorders, without the need for invasive procedures.[1-3] Recent efforts to isolate fetal cells have focused on fetal erythroblasts, because they have a short lifespan, are present in significant numbers in the peripheral blood of early fetuses and have a full complement of nucleogenes.[4-7] Two main problems need to be overcome: first, the low frequency of the fetal cells (1:10^3 to 1:10^8 of maternal nucleated cells)[8-12] and second, the need for an unequivocal marker for the fetal origin of the cells detected.

Numerous methods of cell separation have been developed and enabled fetal cell isolation from maternal blood: fluorescent activated cell sorting (FACS), magnetic activated cell sorting (MACS) and immunogenics.[12-14] Each method has its advantages and disadvantages with respect to efficiency, purity, yield and cost. Of these enrichment techniques, the largest-scale published data concern triple density gradient centrifugation and MACS.[15,16] Enrichment procedures have used monoclonal antibodies to the antigens CD71 and glycophorin A. These antigens were selected because they have been found to be expressed on fetal erythroblasts and CD71 antigen is the one most widely and successfuly used. Unfortunately these antigens are not unique to fetal cells as they are also expressed by maternal nucleated cells.[13] In normal pregnancy hemoglobin production involves two developmental 'switches'; from embryonic ($\zeta_2\varepsilon_2$) to fetal ($\alpha_2\gamma_2$) in the first trimester and to adult ($\alpha_2\beta_2$) at birth.[17,18] Previous studies showed the possibility of identifying erythroblasts enriched from maternal blood by using the fetal γ-globin chain as a marker.[19] However, a very small amount of fetal hemoglobin is also expressed in adults and pregnancy itself causes an increase in maternal cells producing fetal hemoglobin.[20,21] In contrast, embryonic globin chains are unique to fetal erythroblasts and they are therefore potentially the best cell markers to be used in identification of enriched fetal cells from maternal blood. A previous report demonstrated the use of ζ-globin chain as a marker in detecting isolated fetal cells from maternal blood for prenatal diagnosis of thalassemia and sick-

le cell disease.[22] Another report described the use of ε-globin and γ-globin chains in 7 cases in which the maternal sample was taken before and 10 cases in which it was taken after chorionic villus sampling.[23] However, no reports have yet described the distribution and pattern of embryonic hemoglobins (ε and ζ) in a large number of women with normal pregnancies.

Our aim was to investigate the distribution of embryonic and fetal hemoglobin chains in fetal erythroblasts enriched from maternal blood as potential markers for identification of fetal cells and subsequent genetic and chromosomal analysis.

Design and methods

Maternal blood samples (20 mL) were obtained from the antecubital vein from 187 women with singleton pregnancies immediately before chorionic villus sampling (CVS) for fetal karytotyping at 11-14 weeks of gestation. In this study we report findings in patients with normal fetal karyotype. Gestational age was calculated from the date of the last menstrual period and confirmed by the ultrasound examination. Women gave written consent to participate in the study and the blood was collected into lithium heparinized vacutainers (Beckton Dickinson, Franklin Lakes, NJ, USA) and stored at 4°C. Blood was also taken from adult controls (n=20) to assess the false positive rates for each of the three hemoglobin chains. All samples were processed within 24 hours of collection. Triple density gradient centrifugation with Histopaque (Sigma Aldrech Ltd., Poole, England) was carried out as previously described[15] and the middle layer containing the erythroblasts was separated.[15] The isolated cells were then incubated with magnetically labeled CD71 antibody to the transferrin receptor antigen (Miltenyi Biotech, Bergisch Gladbach, Germany) for 15 min at 4°C and enrichment was performed with magnetic cell sorting.[15] In each case four aliquots of the positively selected erythroblasts were cytocentrifuged at 14.3 g for 10 min (Shandon, Frankfurt, Germany), and the cells were cytospun onto four glass slides. One slide was stained with the Kleihauer-Betke stain (GTI, North Patrick Boulevard, Brookfield, Wisconsin, USA) and then counterstained with methylene blue of Giemsa stain (Gurr-Giemsa, BDH Merck Ltd., Poole, England). The remaining three slides were examined for the presence of embryonic (ζ, ε) and fetal (γ) globin chains (Figure 1). Cells were fixed and permeabilized, as previously described,[18] using commercial Fix and Perm reagents (Caltac Burlingame, CA, USA). Slides were then washed in phosphate buffered saline (PBS) solution and incubated with monoclonal fluorescein isothiocyanate (FITC) conjugated fluorescent antibody for the ζ, ε and γ chains. After antibody incubation, the slides were washed in PBS solution, mounted with 4,6-diamidino-2-phenylindole (DAPI) and visualized under a fluorescence microscope (Zeiss Axioskop microscope, Carl Zeiss, Gottingen, Germany). The total number of nucleated cells was counted and the number and percentage of cells positive for the Kleihauer-Giemsa, ζ, ε, and γ-chains were counted and calculated out of the total nucleated cells. At least 100 nucleated cells per slide were counted.

The remaining cells in the positive cell fraction were treated with KCl and fixed with methanol/ glacial acetic acid. Fluorescent *in situ* hybridization (FISH) was carried

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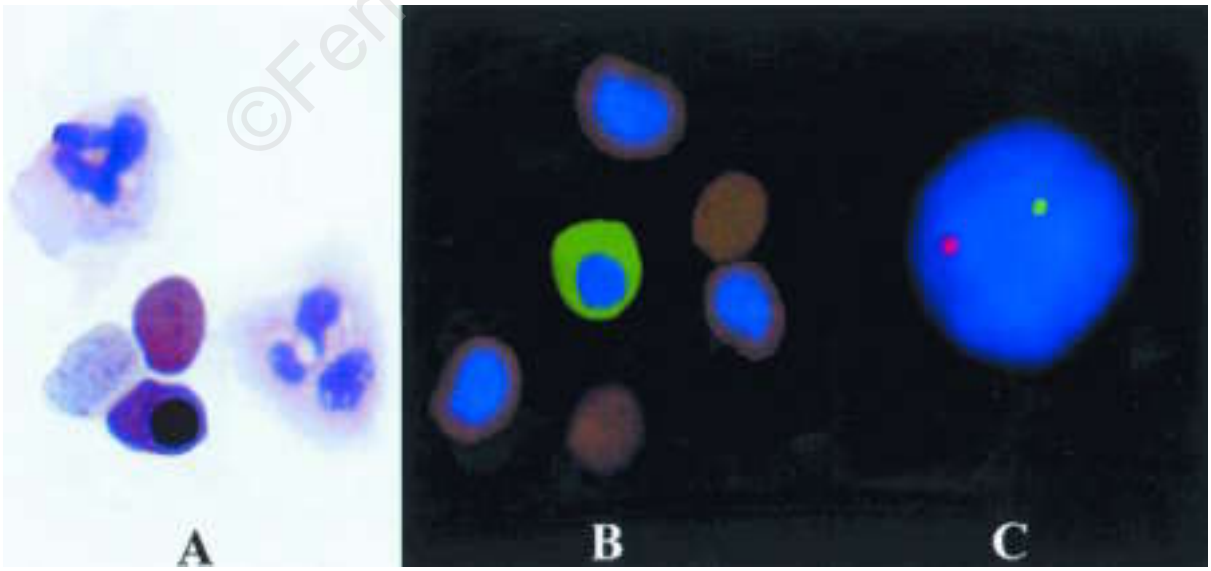

Figure 1. Fetal erythroblasts in maternal blood demonstrated by (A) Kleihauer-Giemsa staining, (B) ε-hemoglobin green fluorescent stain, and (C) FISH for X and Y-signals (red = Y-chromosome, green = X chromosome).

out as previously described[15] using the Vysis multicolor chromosome-specific DNA probe kit (Vysis Inc., Downers Grove, IL., USA) to detect X and Y chromosomes (Figure 1). At least 100 nucleated cells were examined on each slide and the percentages of cells with one signal for the Y chromosome probe, and one, two and three signals for the X chromosome probe were calculated. Only intact cells that were not overlapping were chosen for the analysis. The slides were examined under fluorescence microscope (Zeiss Axioskop microscope, Carl Zeiss, Göttingen, Germany), using the DAPI/FITC/TRITC triple band pass filter set. Image capturing and processing was managed by a Microsoft computerized system (Vysis, USA).

The percentage of positive cells was calculated for each globin chain and comparisons were made between the three chains using Spearman's correlation coefficient. For each globin chain the relation between fluorescent antibody positive erythroblasts (as a percentage of the total nucleated cells) and cells positive for Y-signals was determined by correlation regression analysis. The investigator analyzing these slides was blind to the karyotype results of the cases.

Results

The median maternal age of the 187 women was 33 year (range 16-46) and the median gestation at the time of sampling was 12 weeks (range 11-14). Examination of the enriched cells after staining with the Kleihauer-Giemsa method demonstrated fetal hemoglobin-positive cells in 178 (95%) of the 187 cases; the median percentage of positive cells was 3 (range 0.7-9). Examination of the enriched erythroblasts demonstrated that expression of the ζ chain was observed in 70 (37%) of the cases and the median percentage of positive cells was 0.5% (range 0.2-5%). Expression of the ε chain was observed in 178 (95%) of the cases and the median percentage of positive cells was 1 (range 0.2-7%). Expression of the γ chain was also observed in 178 (95%) of the cases and the median percentage of positive cells was 2% (range 0.5-8%) (Figure 2).

There was a significant association between the percentage of erythroblasts that were positive with the Kleihauer-Giemsa stain and expression of ζ-chain (r=0.58, $p<0.001$), ε-chain (r=0.7, $p<0.001$) and γ-chain (r=0.84, $p<0.001$), respectively (Figure 3). There was also an association between percentage expression of the ζ and ε chains (r=0.59, $p<0.001$), ζ and γ chains (r=0.47, $p<0.001$) and ε and γ chains (r=0.52, $p<0.001$).

In the 187 cases studied, there were 107 pregnancies with male fetuses. In these 107 males, Y-signals were detected in 65 (61%) with a median percentage of positive cells of 3%, and in 42 (39%) of the male cases, Y-signals were absent. In the 80 female pregnancies, no Y-signals were detected.

There was an association between the percentage of erythroblasts that were positive for Y-signals on FISH and expression of ζ-chain (r=0.5, $p<0.001$), ε-chain (r=0.4, $p<0.001$) and γ-chain (r=0.6, $p<0.001$), respectively (Figure 4).

In the adult non-pregnant controls three of the 20 cases demonstrated cells positive for the γ-chain at a percentage of 0.2, 0.3 and 0.7%, but none expressed any cells positive for the ζ or ε chains.

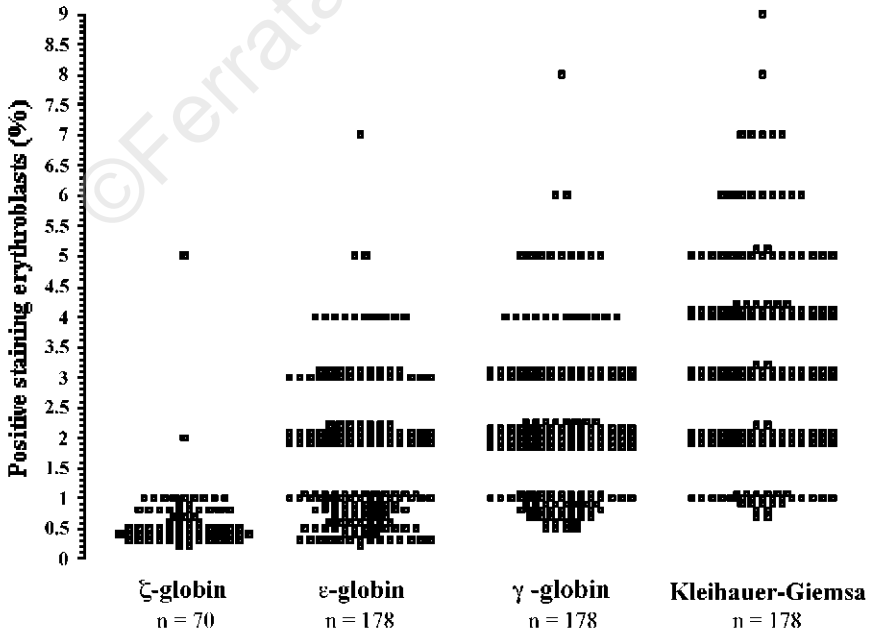

Figure 2. Percentage of fetal erythroblasts in the enriched maternal blood sample positive for the ζ, ε and γ globin chains and for the Kleihauer-Giemsa stain. In total 187 cases were examined; the number of positive cells is given under each stain.

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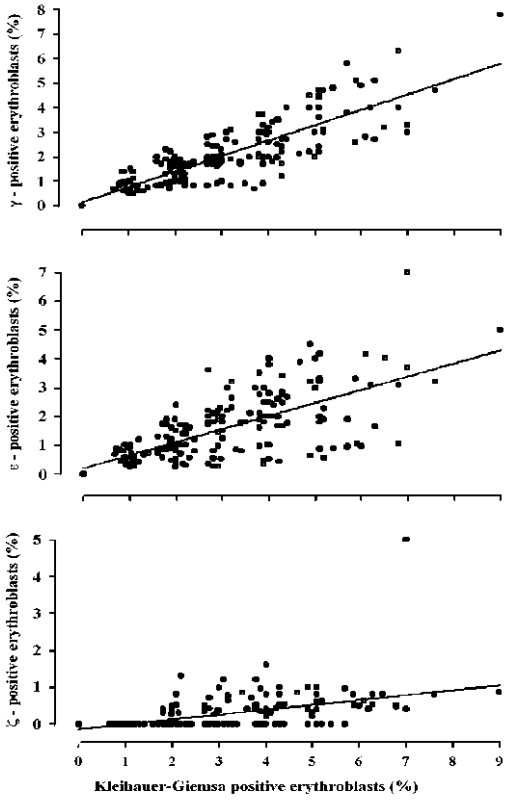

Figure 3. The relationship between the percentage of fetal erythroblasts enriched from maternal blood that stained positive with the Kleihauer-Giemsa stain and γ-chain, ε-chain and ζ-chain.

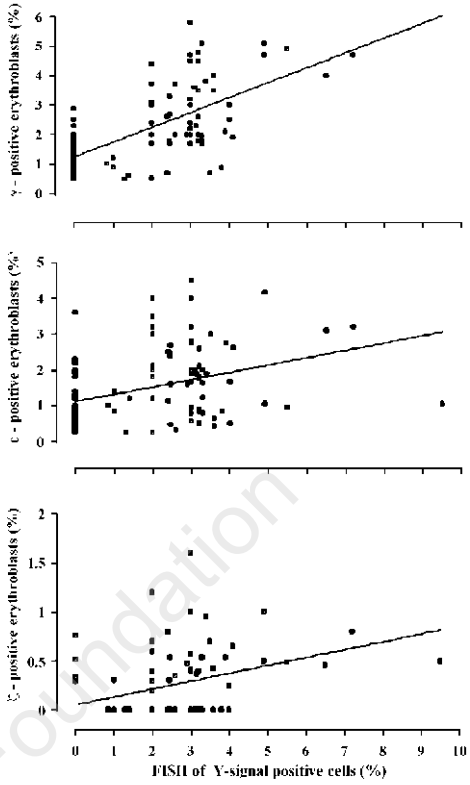

Figure 4. The relationship between the percentage of fetal erythroblasts enriched from maternal blood that stained positive with FISH for Y-signals and γ-chain, ε-chain and ζ-chain.

Discussion

This study has demonstrated the presence of fetal erythroblasts enriched from maternal blood by using embryonic and fetal hemoglobins as fetal cell markers. Our finding agrees with previous reports.[22,23] Cheng *et al.*[22] studied 8 normal cases for the presence of ζ-chain, and Messer *et al.*[23] studied 17 pregnant women, 7 prior to and 10 after chorionic villous sampling, for the presence of ε- and γ-chains. The number of pregnancies examined in these two studies was small, and they were, therefore, not adequate for describing the distribution of embryonic hemoglobins in maternal blood. We, however, studied a larger number of women and were able to determine the variations and pattern of these hemoglobins among the different pregnant women in the first trimester. In our study, about 95% of cells expressed ε and γ chains whereas ζ-chain expression was found in only 37% of cases. Furthermore, the median percentage of expression was 1% for the ε-chain, 2% for the γ-chain and 0.5% for the ζ-chain. The low degree of expression of the ζ-chain is not surprising because our study population was at 11-14 weeks of gestation and by this stage the switch from embryonic to fetal hemoglobin has already occurred. In a previous study involving hemoglobin chain expression in erythroblasts obtained from fetal blood we found that at 12 weeks hemoglobin chain expression was about 45% for the ε-chain, 75% for the γ-chain and 10% for the ζ-chain.[18]

The detection rate of the three globin chains in our study, and in particular the detection of ε-globin chain (95%), might raise the question of possible non-specific staining. However, in our non-pregnant female and male adult control samples, no ε-positive or ζ-positive cells were detected, providing strong evidence of the specificity of the staining and utility of this technique in identifying fetal cells for further analysis. In our enriched samples there were some nucleated cells that stained positive with DAPI but did not stain with either γ, ε, or ζ chains. The origin of these cells could be maternal white cells, fetal erythroblasts that have ceased to express γ, ε, and ζ-chains and contain only α- and β-globin chains, or maternal erythroblasts containing only α- and β-globin chains.

The association between the percentage of erythroblasts that were positive for the Kleihauer-Giemsa stain and expression of the fetal and embryonic hemoglobins is not surprisingly, since the stain identifies acid-resis-

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tant hemoglobin and both fetal and embryonic hemoglobins have this property. Interestingly in 5% of our cases there was no expression of any of the fetal or embryonic haemoglobin chains. This suggests that if enrichment of fetal cells in the maternal circulation is to be used in the future for prenatal diagnosis, in about 5% of women it would still be necessary to resort to the traditional techniques of intrauterine invasive testing.

Although there was an association between the percentage of cells positive for Y-signal FISH and those positive for ε-globin chain, the detection of Y-signals failed in 39% of male cases while the detection of ε-chain failed in 5% of cases. This difference is most likely to be due to the differing technique between FISH and immunostaining. Variations in hybridization efficiency are well-recognized phenomena of FISH,[15,24,25] and various reports have described the technical problems associated with FISH techniques. A variety of factors could influence hybridization efficiency and signal specificity such as enumeration of interphase chromosomes, chromosomal location within the nucleus, cell type, conditions under which the cells are manipulated (e.g., magnetic/flow sorting), sample fixation, cell permeability, and probe size/complexity.[26-31] Recently Samura *et al.*[32] studied the origin of γ-positive erythroblasts enriched from maternal blood by DNA polymorphism in pregnancies with female fetuses that showed two X-signals on FISH. They found that DNA polymorphism determined most of the γ-positive erythroblasts to be fetal in origin. The study confirmed the technical problems associated with FISH and raised the issue of using alternative techniques such as polymerase chain reaction (PCR) to obtain accurate chromosomal analysis. Potentially the ε-chain can be used as a marker to identify fetal erythroblasts in maternal blood, followed by micromanipulation of these cells and PCR analysis. This remains to be seen in the future.

Previous studies have successfully isolated fetal cells from maternal blood and detected chromosomal abnormalities in these enriched cells.[16,33-35] However, in these studies, the isolated erythroblasts were identified by the Giemsa stain, and therefore maternal as well as fetal erythroblasts were stained, with false identification of cells as fetal in origin. Furthermore, the FISH analysis , in these studies showed a poor yield of Y-chromosomes and other chromosomal analyses. The investigators concluded that most of the enriched erythroblasts from maternal blood were maternal in origin. Using non-specific stains for morphologic identification, they obtained inaccurate assessment of fetal cell number and frequency. Their lower number of cells with Y-signals on FISH could be related to the technical problems associated with the FISH techniques described above. Our study, however, is a further step in fetal cell detection. We used a unique marker of fetal cells that results in definite fetal cell identification. These markers enabled an accurate estimation of fetal cell number in maternal blood in the first trimester of pregnancy. The individual fetal cells that are positive for the embryonic markers can, potentially, then be further analyzed by micromanipulation and single cell PCR.

We demonstrated the distribution of embryonic hemoglobin chains in the first trimester of pregnancy and the potential use of these chains as cell markers in the isolation of fetal erythroblasts from the maternal circulation. Our findings suggest that attempts to identify fetal erythroblasts in maternal blood at 11-14 weeks should focus on the use of markers for the ε-chain, since at this period of gestation expression of the ζ-chain is very low and expression of the γ-chain is not confined to fetal erythroblasts. In combination with other techniques such as micromanipulation, PCR analysis, better FISH techniques and automated image analysis, these markers have the potential to be used in the future to improve detection and analysis of fetal erythroblasts for prenatal diagnosis.

Contributions and Acknowledgments

AMR: contributor of the concept and design of the study, principal investigator, performed all the experimental work, data analysis and statistical work, and wrote the manuscript. HH: supervised the experimental work, contributed to the revision of the manuscript. FF: contributed to the revision of the manuscript. KHN: main supervisor of the study, supplied blood samples, reviewed data analysis, contributed to the writing and revision of the manuscript.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential implications for clinical practice

The embryonic hemoglobin ε-chain has the potential to be used in the future as a unique fetal cell marker in combination with other techniques to improve detection and analysis of enriched fetal erythroblasts from maternal blood for prenatal diagnosis of genetic and chromosomal abnormalities in the first trimester of pregnancy.

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