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Fetal Gender Determination and BclI Polymorphism Using Nucleated Erythrocytes in Maternal Blood

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SUMMARY   This study demonstrated determination of fetal gender from nucleated red blood cells (NRBCs) in maternal blood and attempted to apply prenatal diagnosis of hemophilia A using BclI DNA polymorphism. Venous blood was drawn from 20 pregnant women, and NRBCs were recovered by magnetic activated cell sorting and anti-GPA (glycophorin A) immunostaining. After microdissector isolation of the NRBCs, primer extension preamplification (PEP) and nested PCR of the amelogenin gene were performed to determine fetal gender. We also performed PEP and nested PCR of BclI polymorphism to verify the validity of prenatal diagnosis of hemophilia A. DNA amplification was achieved in 107 cells (51.9%) and fetal gender determined with 65.0% accuracy. Unfortunately, we could not verify the validity within the scope of this study. However, in a larger number of cases that are informative in BclI polymorphism, we will be able to identify patients affected by hemophilia A using fetal NRBCs in maternal blood.   **(J Histochem Cytochem 53:323–327, 2005)**

CURRENTLY AVAILABLE METHODS of obtaining fetal material for prenatal diagnosis present significant procedure-related risks to both the fetus and the mother. Many researchers have made efforts to obtain fetal materials by noninvasive means. Among those methods, fetal cells, especially nucleated erythrocytes [nucleated red blood cells (NRBCs)] in maternal peripheral blood, have been the focus of research during the past 15 years.

Various immunological traits have been tried to enrich fetal NRBCs. We used magnetic activated cell sorting (MACS) for CD71 (transferrin receptor) of NRBCs. After positive MACS with anti-CD71 antibody, we could expect that the magnetically labeled cell suspension eluted from the MACS column contains NRBCs, activated lymphocytes, and macrophages. Because cells of erythroid lineage have glycophorin A (GPA) as a cell membrane antigen, we used immunostaining with an anti-GPA antibody to differentiate fetal NRBCs in cell suspension, which eluted after MACS for CD71. It has been well established that this combination of antigens is excellent for isolation of nucleated erythrocytes (Bianchi et al. 1990,1992; Price et al. 1991; Wachtel et al. 1991; Ganshirt-Ahlert et al. 1992; Reading et al. 1995). However, even after separation with monoclonal antibodies, maternal cell contamination is unavoidable. Therefore, we picked up a single cell with a micromanipulator (Takabayashi et al. 1995).

The problem is that NRBCs in maternal peripheral blood are of both fetal and maternal origin (Ganshirt et al. 1994; Slunga-Tallberg et al. 1995,1996; von Eggeling et al. 1997; Holzgreve et al. 1998; Troeger et al. 1999). However, despite the fact that nucleated erythrocytes in maternal peripheral blood are of fetal and maternal origin, many researchers have claimed that they can diagnose various disorders using NRBCs in maternal peripheral blood, such as hemoglobinopathies (Di Naro et al. 2000), Rhesus type, and Duchenne muscular dystrophy (Sekizawa et al. 1996a,b).

Therefore, we focused on the possibility of diagnosis of hemophilia A using fetal nucleated erythrocytes in maternal peripheral blood. In Korea, PCR-based

analysis of the BclI/intron 18 and St14 VNTR polymorphisms is useful in carrier detection and prenatal diagnosis of hemophilia A, and the observed heterozygosity for BclI/intron 18 polymorphism was 21.0% (Choi et al. 2000). Because it is very difficult to apply St14 VNTR analysis in single-cell PCR, we tried BclI polymorphism to diagnose hemophilia A.

In this study, we first tried to examine the efficacy of a noninvasive method by using fetal NRBCs in maternal peripheral blood to determine fetal gender. Second, we aimed to check the validity to diagnose hemophilia A by BclI polymorphism using fetal cells in maternal blood.

Materials and Methods

Peripheral blood samples (20 ml) were collected in EDTA tubes from 20 healthy women with uncomplicated singleton pregnancies (10 male, 10 female fetuses) between 10 and 22 weeks of gestation. This patient population included nine primigravidas and 11 multigravidas referred to our antenatal testing unit for amniocentesis or chorionic villus sampling (CVS) because of advanced maternal age and abnormal triple screen results. All patients were Rh positive. The samples were collected before amniocentesis or CVS was performed.

Isolation and Enrichment of Nucleated Cells

The blood samples were diluted with a double volume of PBS. Nucleated cells were isolated by density gradient centrifugation using Ficoll-Paque (Pharmacia Biotech; Uppsala, Sweden). Centrifugation was performed at $400 \times g$ for 30 min. The layer of cells between plasma and Ficoll-Paque, in which most mononucleated cells were recovered, was washed with PBS, pH 7.2, and centrifuged at $300 \times g$ for 10 min. After the washing procedure was repeated and the supernatant decanted, the cell pellet was resuspended in PBS to allow counting of the total number of recovered cells.

After centrifugation and resuspension of the pellet in PBS [supplemented with 0.5% bovine serum albumin (BSA) and 2 mM EDTA], the samples were incubated for 15 min at 4C with a 1:10 solution of monoclonal antibodies (CD 71; Miltenyi Biotec, Bergisch Gladbach, Germany) and inactivated plasma. Separation was achieved with the mini-MACS system (Miltenyi Biotec) according to the manufacturer's recommendations.

Slide Preparation and Immunophenotyping

After mini-MACS separation, in theory the eluted cell suspension would contain NRBCs, activated lymphocytes, and macrophages. We tried to separate NRBCs from the remaining cells using anti-GPA antibody. Therefore, we made slides for microscopic examination by cytocentrifugation (Shandon Southern; Runcorn, U.K.). The slides were fixed in cold acetone for 5 min. Anti-GPA (mouse anti-human glycophorin A) monoclonal antibody (DAKO; Carpinteria, CA) was used to identify NRBCs by 3-amino-9-ethyl-carbazole immunostaining (DAKO). Hematoxylin counterstaining was used for morphological characterization of the cells. Finally,

the immunophenotyped cells were classified under a light microscope. The coordinates of GPA-positive nucleated erythrocytes on the slides were recorded and the cells photographed.

Single-cell Isolation by Microdissector and Fine Needle

Single NRBCs, recognized as red by anti-GPA immunocytochemistry, were collected with a 30-G needle and microdissector (Figure 1). Each NRBC was transferred to its own tube containing 5 µl distilled water and then snap-frozen in liquid nitrogen. Protein digestion was carried out for 2 hr at 60C by adding 5 µl proteinase K (500 µg/ml)/buffer mixture (10 mM Tris-HCl, pH 8.3, 50 mM KCl). After complete digestion, proteinase K was inactivated by heating to 94C for 2 min.

Amplification of DNA by Primer Extension Preamplification

Primer extension preamplification (PEP) was performed as described by Zhang et al. (1992). Briefly, the initial reaction volume containing the digested single cell was brought to a final volume of 60 µl with 33 µM totally degenerated 15-mer primer (Operon Technologies; Alameda, CA), PCR buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 100 µM dNTPs, and 5 U Taq polymerase (Takara; Tokyo, Japan). Fifty cycles were carried out in a PTC 200 thermocycler (MJ Research; Cambridge, MA). Each cycle consisted of 1 min denaturation at 92C, 2 min annealing at 37C, heating to 55C at 0.1C/sec, and 4 min extension at 55C. After PEP, the PCR product was stored at 4C as a template for nested PCR.

Nested PCR of Amelogenin Loci and BclI Polymorphic Loci

We used nested PCR amplification of the amelogenin gene on chromosomes X and Y for gender determination of the samples (Schaaff et al. 1996). The following procedure was used for PCR: 45 µl of PCR mix was added to each 5 µl PEP aliquot, resulting in a total reaction volume of 50 µl and final concentrations of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 0.2 mM of each dNTP, 400 nM of each outer primer [AMG 3 (5′-CTT CCC AGT TTA AGC TCT GAT G-3′), AMG 4 (5′-CCT TGC TCA TAT TAT ACT TGA C-3′)], and 1 U Taq polymerase. Samples were heated at 94C for 5 min to ensure DNA denaturation, followed by 30 cycles of denaturation at 94C for 30 sec, annealing at 52C for 60 sec, and extension at 72C for 60 sec. Final extension at 72C was allowed for 10 min. For nested amplification, 2 µl of amplified product was transferred to a new reaction tube containing 23 µl of fresh PCR mix. This mix was the same as outer PCR except that it contained 400 nM of each inner primer [AMG 5 (5′-CTC AGG GAG GTT CCA TGA-3′), AMG 6 (5′-TGA GAA AAC CAG GGT TCC-3′)] in place of the outer primers, and the $MgCl_2$ concentration was increased to 2.5 mM. The amplification cycle of inner PCR included denaturation at 94C for 30 sec, annealing at 56C for 30 sec, and extension at 72C for 60 sec. Final extension was performed at 72C for 10 min. Amplification products were electrophoresed on 2%

Figure 1 After MACS and anti-GPA antibody immunostaining (**A**), single cells can be harvested with a micro-dissector and a 30-G needle (**B**).
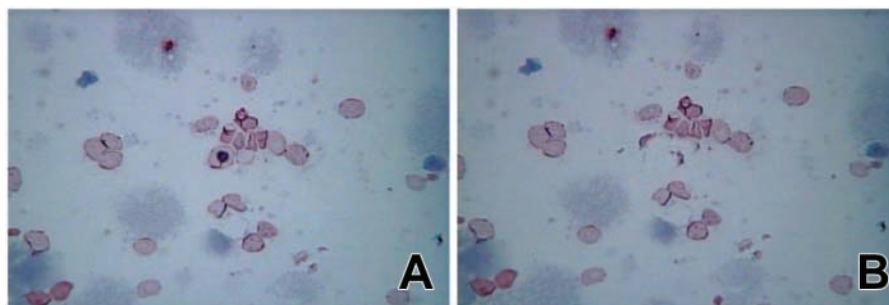
agarose gel in 1 × tris borate EDTA with ethidium bromide and visualized using an ultraviolet transilluminator. A cell was considered to be genotypically female if one band of 323 base pairs (bp) was present and male if a band of 142 bp was also present.

BclI polymorphism (Snabes et al. 1994) was performed for seven pregnant women. The slightly modified PCR procedure was as follows. A 45-µl PCR mix was added to each 5-µl PEP aliquot, resulting in a total reaction volume of 50 µl and final concentrations of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl$_2$, 0.25 µM of each dNTP, 0.25 mM of each outer primer [HA1F (5′-CTA CCT GGC TTA GTA ATG GCT C-3′), HA1R (5′-AAA GGA ATA AAT TCC TTT TCC C-3′)], and 1.25 U Taq polymerase. Samples were heated at 92C for 3 min to ensure DNA denaturation, followed by 20 cycles of denaturation at 92C for 30 sec, annealing at 49C for 30 sec, and extension at 72C for 30 sec. Final extension at 72C was performed for 3 min. For nested amplification, 2 µl of amplified product was transferred to a new reaction tube containing 23 µl of fresh PCR mix. This mix contained 0.25 mM of each inner primer [HA2F (5′-ATC AAA GGA TTC GAT GGT ATC T-3′), HA2R (5′-TTT CCT TTT TAG CAA TTT TTC T-3′)] in place of the outer primers. After initial denaturation at 92C for 3 min, 30 cycles of amplification were carried out for inner PCR (92C for 30 sec, 46C for 30 sec, and 72C for 30 sec for denaturation, annealing, and extension, respectively). Final extension was performed at 72C for 3 min. BclI (30 U) was added directly to the PCR product and after 1 hr at 50C, 10-µl aliquots were electrophoresed on a 2% agarose gel.

Results

The first part of this study aimed to examine the efficacy of a method using fetal cells in maternal peripheral blood by determining fetal gender using the amelogenin gene from 20 pregnant women.

In total, 206 cells were obtained from peripheral blood of 20 pregnant women. Among these, 107 cells (51.9%) could be amplified by PEP and nested PCR of the amelogenin gene.

In each of 20 cases, 1–25 NRBCs were screened with the amelogenin system for gender determination (Table 1). Gender was ascertained in each case by karyotyping of chorionic villi or amniocytes.

In this study, we assigned the fetus as male if we could find any single cell that showed a band corre-

sponding to the Y-chromosome allele in amelogenin PCR. According to this rule, we correctly identified 8/10 male fetuses and 5/10 female fetuses. Combining these results, the accuracy of gender determination in our approach was 65.0%.

The second part of this study aimed to check the validity of diagnosing hemophilia A by BclI polymorphism using fetal NRBCs in maternal blood. We selected 85 NRBCs from seven pregnant women, of which 47 cells (55.3%) could be amplified by PEP/nested PCR of BclI polymorphism. However, only one of the seven pregnant women showed a difference between fetus (amniotic fluid) and mother (peripheral blood) in restriction fragment length polymorphism pattern. That is to say, BclI polymorphism was informative in only one pregnant woman. Because of lack of informative cases using BclI polymorphism in this study, we could not be sure whether prenatal diagnosis of hemophilia A by fetal NRBCs in maternal peripheral

Table 1 Nucleated erythrocytes separated from 16 pregnant women amplified by PEP and amelogenin nested PCR

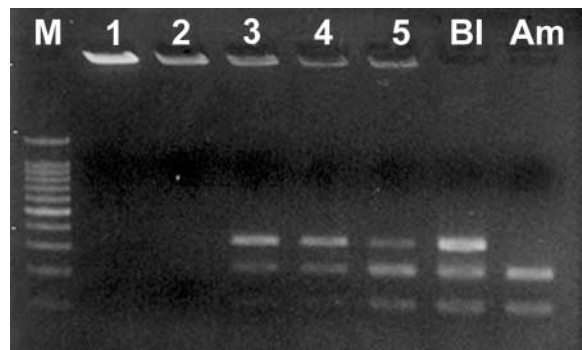| Case number	Cells retrieved, n	Cells amplified, n	Female band	Male band	Gender assigned	True gender
1	5	3	3	0	F	F
2	18	3	3	0	F	F
3	8	2	2	0	F	F
4	10	10	10	0	F	F
5	7	1	1	0	F	M
6	19	8	7	1	M	M
7	6	6	3	3	M	M
8	9	9	0	9	M	M
9	25	24	6	18	M	M
10	20	2	0	2	M	M
11	16	6	1	5	M	M
12	17	7	5	2	M	M
13	5	5	1	4	M	F
14	14	8	6	2	M	F
15	7	7	0	7	M	F
16	2	2	0	2	M	F
17	2	1	1	0	F	M
18	1	1	1	0	F	F
19	9	1	0	1	M	F
20	6	1	0	1	M	M
Total	206	107				

PEP, primer extension preamplification.

Figure 2 Agarose gel electrophoresis of BclI polymorphism results from a male fetus. Three cells (Lanes 3–5) of five show the same pattern as the mother (Bl), despite coming from male fetuses (Am). DNA in Lanes 1 and 2 could not be amplified.

blood is applicable. However, in this case, in spite of a male-bearing pregnancy, three cells showed a female-specific band pattern in amelogenin PCR. In addition, these three cells showed the same band pattern as maternal cells in BclI polymorphism (Figure 2). Therefore, we could conclude that these three cells were of maternal origin.

Discussion

Because of the rarity of fetal NRBCs in maternal peripheral blood, it has been a challenge to enrich fetal cells from maternal peripheral blood and then analyze them for genetic disorders.

In this study, we used CD71 antibody against the transferrin receptor, which has been widely employed. In addition to MACS with the anti-CD71 antibody, we further tried to differentiate fetal NRBCs from other cells by anti-GPA antibody and by single-cell pick-up with a micromanipulator.

However, this study was fraught with considerable amplification failure (48.1%). There appeared to be several explanations for amplification failure. First, some template degradation may have occurred during the course of the enrichment and staining procedures (Reading et al. 1995; Cheung et al. 1996). Second, microdissection of cells fixed on slides can induce mechanical DNA breakage and thus loss of amplification from one or two alleles (Cheung et al. 1996). In these situations, however, analysis of several cells could avoid misdiagnosis. Third, it is probably to some extent related to cell loss during the micromanipulation, because the final transfer of the picked cell to the PCR tube is not under visual control. Laser-assisted micromanipulation and automatic transfer directly into the PCR tube (Schutze and Clement-Sengewald 1994) might circumvent cell loss and mechanical DNA breakage. Finally, although we used PEP, it is impossible to

amplify the target sequences every time because of significant locus-dependent variation in amplification efficiency (Sekizawa et al. 1996b).

Despite these problems, we could correctly assign fetal gender in 13/20 (65%) pregnant women. This result is comparable to those of previous studies (Sekizawa et al. 1996a,b; von Eggeling et al. 1997).

Because we used heterozygous alleles (amelogenin gene and BclI polymorphism) and whole-genome amplification, we expected to have allelic drop-out (ADO).

As many researchers (von Eggeling et al. 1997; Garvin et al. 1998; Hahn et al. 1998) have observed, ADO is more likely to occur after whole-genome amplification. Nevertheless, Hahn et al. (1998) and Garvin et al. (1998) have recommended examination of multiple single cells and multiplex PCR for simultaneous identification using microsatellite loci known to be informative for solving the problem of ADO. Accordingly, we performed PCR in 47 cells and performed PCR for the amelogenin gene and BclI polymorphism sequentially to avoid ADO. Nevertheless, only one male-bearing pregnancy was informative in BclI polymorphism, and because of the relatively high failure rate in amplification, we cannot report the exact number of cells showing ADO.

Interestingly, three cells in this case showed a female-specific band in amelogenin PCR despite a male-bearing pregnancy and showed the same band pattern as that of the mother in BclI polymorphism as well. Combining these results, we could conclude that these three cells were of maternal origin. This finding also gives evidence that NRBCs in maternal blood are of both fetal and maternal origin, and it is comparable to those of previous studies (Ganshirt et al. 1994; Slunga-Tallberg et al. 1995,1996; von Eggeling et al. 1997; Holzgreve et al. 1998; Troeger et al. 1999).

It was not possible within the scope of this study to obtain large-scale results from BclI polymorphism, and therefore, because of the lack of informative cases, we could not demonstrate the validity of prenatal diagnosis of hemophilia A in our approach. However, with a larger number of cases that are informative in BclI polymorphism, we could identify patients affected by hemophilia A using fetal NRBCs in maternal blood.

In summary, our data demonstrate that our experimental design is valid and that if we apply our approach to more cases that are informative in BclI polymorphism, it will be possible to use fetal NRBCs in maternal blood for prenatal diagnosis of hemophilia A.

Literature Cited

Bianchi DW, Flint AF, Pizzimenti MF, Knoll JH, Latt SA (1990) Isolation of fetal DNA from nucleated erythrocytes in maternal blood. Proc Natl Acad Sci U S A 87:3279–3283

Bianchi DW, Mahr A, Zickwolf GK, Houseal TW, Flint AF, Klinger KW (1992) Detection of fetal cells with 47,XY,+21 karyotype in maternal peripheral blood. Hum Genet 90:368–370

Cheung MC, Goldberg JD, Kan YW (1996) Prenatal diagnosis of sickle cell anaemia and thalassaemia by analysis of fetal cells in maternal blood. Nat Genet 14:264–268

Choi YM, Hwang D, Choe J, Jun JK, Kim EJ, Moon SY, Cho S (2000) Carrier detection and prenatal diagnosis of hemophilia A in a Korean population by PCR-based analysis of the BclI/intron 18 and St14 VNTR polymorphisms. J Hum Genet 45:218–223

DiNaro E, Ghezzi F, Vitucci A, Tannoia N, Campanale D, D'Addario V, Holzgreve W, et al. (2000) Prenatal diagnosis of beta-thalassaemia using fetal erythroblasts enriched from maternal blood by a novel gradient. Mol Hum Reprod 6:571–574

Ganshirt D, Garritsen H, Miny P, Holzgreve W (1994) Fetal cells in maternal circulation throughout gestation. Lancet 343:1038–1039

Ganshirt-Ahlert D, Burschyk M, Garritsen HS, Helmer L, Miny P, Horst J, Schneider HP, et al. (1992) Magnetic cell sorting and the transferrin receptor as potential means of prenatal diagnosis from maternal blood. Am J Obstet Gynecol 166:1350–1355

Garvin AM, Holzgreve W, Hahn S (1998) Highly accurate analysis of heterozygous loci by single cell PCR. Nucleic Acids Res 26:3468–3472

Hahn S, Garvin AM, Di Naro E, Holzgreve W (1998) Allele dropout can occur in alleles differing by a single nucleotide and is not alleviated by preamplification or minor template increments. Genet Test 2:351–355

Holzgreve W, Ghezzi F, Di Naro E, Ganshirt D, Maymon E, Hahn S (1998) Disturbed feto-maternal cell traffic in preeclampsia. Obstet Gynecol 91(5 pt 1):669–672

Price JO, Elias S, Wachtel SS, Klinger K, Dockter M, Tharapel A, Shulman LP, et al. (1991) Prenatal diagnosis using fetal cells isolated from maternal blood by multiparameter flow cytometry. Am J Obstet Gynecol 165(6 pt 1):1731–1737

Reading JP, Huffman JL, Wu JC, Palmer FT, Harton GL, Sisson ME, Keyvanfar K, et al. (1995) Nucleated erythrocytes in maternal blood: quantity and quality of fetal cells in enriched populations. Hum Reprod 10:2510–2515

Schaaff F, Wedemann H, Schwinger E (1996) Analysis of sex and ΔF508 in single amniocytes using primer extension preamplification. Hum Genet 98:158–161

Schutze K, Clement-Sengewald A (1994) Catch and move—cut or fuse. Nature 368:667–669

Sekizawa A, Kimura T, Sasaki M, Nakamura S, Kobayashi R, Sato T (1996b) Prenatal diagnosis of Duchenne muscular dystrophy using a single fetal nucleated erythrocyte in maternal blood. Neurology 4:1350–1353

Sekizawa A, Watanabe A, Kimura T, Saito H, Yanaihara T, Sato T (1996a) Prenatal diagnosis of the fetal RhD blood type using a single fetal nucleated erythrocyte from maternal blood. Obstet Gynecol 87:501–505

Slunga-Tallberg A, el-Rifai W, Keinanen M, Ylinen K, Kurki T, Klinger K, Ylikorkala O, et al. (1995) Maternal origin of nucleated erythrocytes in peripheral venous blood of pregnant women. Hum Genet 96:53–57

Slunga-Tallberg A, el-Rifai W, Keinanen M, Ylinen K, Kurki T, Klinger K, Ylikorkala O, et al. (1996) Maternal origin of transferrin receptor positive cells in venous blood of pregnant women. Clin Genet 49:196–199

Snabes MC, Chong SS, Subramanian SB, Kristjansson K, DiSepio D, Hughes MR (1994) Preimplantation single-cell analysis of multiple genetic loci by whole-genome amplification. Proc Natl Acad Sci USA 91:6181–6185

Takabayashi H, Kuwabara S, Ukita T, Ikawa K, Yamafuji K, Igarashi T (1995) Development of non-invasive fetal DNA diagnosis from maternal blood. Prenat Diagn 15:74–77

Troeger C, Zhong XY, Burgemeister R, Minderer S, Tercanli S, Holzgreve W, Hahn S (1999) Approximately half of the erythroblasts in maternal blood are of fetal origin. Mol Hum Reprod 5:1162–1165

von Eggeling F, Michel S, Gunther M, Schimmel B, Claussen U (1997) Determination of the origin of single nucleated cells in maternal circulation by means of random PCR and a set of length polymorphisms. Hum Genet 99:266–270

Wachtel S, Elias S, Price J, Wachtel G, Phillips O, Shulman L, Meyers C, et al. (1991) Fetal cells in the maternal circulation: isolation by multiparameter flow cytometry and confirmation by polymerase chain reaction. Hum Reprod. 6:1466–1469

Zhang L, Cui X, Schmitt K, Hubert R, Navidi W, Arnheim N (1992) Whole genome amplification from a single cell: implications for genetic analysis. Proc Natl Acad Sci USA 89:5847–5851