
Accuracy of Fetal Gender Determination by Analysis of DNA in Maternal Plasma, Akihiko Sekizawa,* Tetsuro Kondo, Mariko Iwasaki, Akira Watanabe, Masatoshi Jinbo, Hiroshi Saito, and Takashi Okai (Department of Obstetrics and Gynecology, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan; * author for correspondence: fax 81-33784-8355, e-mail sekizawa@d8.dion.ne.jp)

In 1997, Lo et al. (1) first described the presence of fetal DNA in maternal plasma and serum. Cell-free DNA offered a new source of fetal genetic material for noninvasive prenatal diagnosis. They developed a real-time, quantitative PCR assay to measure the concentration of fetal DNA and analyzed SRY, a single-copy Y-chromosome-specific sequence, to quantify the number of genome-equivalents per milliliter of blood when a woman carries a male fetus (2). The results revealed that the concentrations of fetal DNA in maternal plasma DNA during the first and third trimesters were 3.4% and 6.2%, respectively. They estimated a mean of 25.4 copies of fetal DNA circulates per milliliter of maternal plasma samples during early pregnancy. They also demonstrated that after delivery, cell-free fetal DNA is cleared very rapidly from the maternal circulation, with a half-life in the order of minutes (3). Thus, the relatively high concentration of fetal cell-free DNA suggests that extensive and time-consuming fetal DNA enrichment procedures would not be necessary when using fetal DNA from maternal plasma for diagnostic purposes.

Although the potential of fetal cell-free DNA analysis for the prenatal diagnosis of fetal gender (2) and rhesus D status (4, 5) has been presented, the accuracy of prenatal diagnosis has not been described. If fetal gender could be determined, the number of invasive procedures required to determine X-linked genetic disorders would be reduced. The present study evaluated the diagnostic accuracy of fetal gender determination using cell-free DNA from maternal plasma obtained at early gestation.

Pregnant women (n = 302), carrying a single fetus between 7 and 16 weeks of gestation, who attended Showa University Hospital between February 1999 and September 2000 gave their written informed consent to participate in this study. Approval for this study was obtained from the ethics committee of the university. None of the women had vaginal bleeding. Fetal heart movements were detected in all of the women by transvaginal ultrasonography.

Maternal blood samples (10 mL) collected into tubes containing EDTA were separated by centrifugation at 3000g within 3 h. Plasma was transferred into plain polypropylene tubes and again separated by centrifugation at 3000g. The supernatant was collected into fresh tubes and stored at −20 °C until further processing.

DNA was extracted from 1.5 mL of plasma samples using QIAamp Blood Mini Kit (Qiagen) according to the “blood and body fluid protocol” with minor modifications. DNA was eluted from columns with 50 µL of water.

Although a real-time quantitative PCR assay using an ABI PRISM 7700 Sequence Detector (Applied Biosystems) for maternal plasma DNA analysis has been reported by several investigators (2, 4, 6, 7), we used a LightCycler (Roche Diagnostics), which is a reliable method of DNA quantification (8). The Y-chromosome-specific sequence, DYS-14, in male fetuses was used as a molecular marker to quantify fetal cell-free DNA (9). The PCR system for the DYS-14 sequence consisted of the amplification primers DYS14-713F (5’-CAT CCA GAG CGT CCC TGG-3’), DYS14-880R (5’-TTA CCC TTT GCT CCC CAA A3’), and the dual-labeled fluorescent probe DYS14-883T (5’-FAM-CGA AGC GAT GCC CAT-3’). The fluorogenic PCR reactions were prepared according to the manufacturer’s instructions in a reaction volume of 20 µL with all components except the fluorescent probe and amplification primers obtained from a reagent set (LightCycler-Fast Start DNA Master Hybridization Probes; Roche Diagnostics). The extracted plasma DNA (13.1 µL) was used as a template for each reaction. Thermal cycling was initiated with denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 57 °C for 20 s, and extension at 72 °C for 20 s. The number of copies of male DNA present in the plasma sample was determined by comparison with a calibration dilution curve of male genomic DNA. The number of copies used for conversion was 6.6 pg, as described previously (2).

Amplification data were analyzed using LightCycler
software (Roche Diagnostics), and the concentration of the DYS14 sequence was calculated. Fetal gender was determined according to the presence or absence of the DYS14 sequence in a blinded manner.

Strict precautions were taken against contamination, and multiple negative water blanks were included in every analysis. A female staff member performed all procedures, including sample preparation, DNA extraction, and PCR amplification. Fetal gender was confirmed at delivery or through the cytogenetic results of amniocentesis. Results were analyzed statistically using Excel 2000 (Microsoft).

Copies of the DYS14 sequence were determined by comparison with a calibration curve of serially diluted, male genomic DNA. The relationship was linear when the threshold cycle was plotted against the input target quantity, with the latter plotted on a common logarithmic scale. The correlation coefficient of the calibration curve ranged from 0.956 to 1.000. The calculated day-to-day CV of the system starting from DNA extraction followed by quantitative PCR was 5.3% (n = 7). We added 1000 copy equivalents of male DNA (6.6 ng of whole blood DNA) into the plasma of nonpregnant women and evaluated DNA recovery after extraction. The recovery rate of male DNA averaged 66.0%.

We analyzed a total 302 maternal plasma samples in a blinded manner (Table 1); 143 of those were carrying male fetuses, and 159 were carrying females. The DYS14 sequence was detected by PCR in 97.2% (139 of 143) of pregnant women bearing male fetuses. False-negative results were found in four pregnant women at 9 (two mothers), 11, and 13 weeks. The results of a reanalysis of the plasma samples from these pregnant women corresponded to the correct fetal gender.

The overall sensitivity of determination of male gender from maternal plasma was 97.2%, the specificity was 100%, the positive predictive value was 100%, and the negative predictive value was 97.5%.

The quantitative findings for the DYS14 sequence in mothers bearing male fetuses are shown in Fig. 1. At 7 weeks of gestation, the DYS14 sequence was detected in eight of eight pregnant women, and the mean concentration of fetal DNA was 28.0 ± 17.7 genome-equivalents per milliliter. Between 7 and 16 weeks of gestation, the mean concentration of fetal DNA was 37.5 ± 32.1 genome-equivalents per milliliter.

Fetal gender is detectable through analyzing maternal plasma at early gestation. The false-negative samples yielded the correct results after reanalysis. A low concentration of fetal DNA or the relatively low recovery rate of DNA extraction might have been responsible for the false negatives. A more reliable DNA extraction procedure should improve the accuracy of our procedure, and if all analyses are performed in duplicate, results may be improved.

Defining fetal gender from maternal plasma could be useful in the management of pregnant women who are heterozygous carriers of X-linked genetic disorders. When testing reveals that a fetus is female, the pregnant woman would be free from risk for an affected baby. Noninvasive prenatal gender determination would reduce the number of invasive procedures by one-half.

The mean concentration of fetal DNA in 143 plasma samples from mothers bearing male fetuses was 37.4 genome-equivalents per milliliter, which is a little higher than reported previously (2). Even at 7 weeks of gestation, the mean fetal DNA concentration in maternal plasma

<table>
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<tr>
<th>Week no.</th>
<th>Total</th>
<th>Male</th>
<th>Female</th>
<th>Accurate diagnosis</th>
<th>False positives</th>
<th>False negatives</th>
<th>Fetal DNA concentrations</th>
<th>Mean</th>
<th>SD</th>
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<td>5</td>
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<td>28.0 ± 17.7</td>
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<td>6</td>
<td>7</td>
<td>13</td>
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<td>0</td>
<td>36.7 ± 31.9</td>
<td>38.5</td>
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<td>24</td>
<td>20</td>
<td>42</td>
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<td>31</td>
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<td>37.4 ± 32.1</td>
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<td>159</td>
<td>298</td>
<td>0</td>
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was $>20$ genome-equivalents per milliliter, and the amount of circulating DNA in maternal plasma would be sufficient to analyze fetal gender.

In conclusion, this is the first description of the diagnostic accuracy of fetal gender determination from a large number of maternal plasma samples obtained at early gestation. Because this test is noninvasive and highly accurate, it can be used as a valuable first step in various clinical settings.

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References


Cryoglobulins Interfere with Platelet Counts by Optical and Impedance Methods but not with the CD61 Immunoplatelet Count, Nicolas von Ahlsen, Colin Stephen Scott, Joachim Riggert, and Michael Oellerich (Departments of 1 Clinical Chemistry and 2 Transfusion Medicine, Georg-August-University, Robert-Koch-Strasse 40, 37075 Goettingen, Germany; 3 Abbott Diagnostika GmbH, 65205 Wiesbaden-Delkenheim, Germany; 4 author for correspondence: fax 49-551-39-12504, e-mail nahsen@gwdg.de)

Automated complete blood counts (CBCs) use supplementary warnings (flags) to provide alerts for the existence of hematologic abnormalities (1). We observed on several occasions platelet-count interference by cryo-globulins, where the impedance and optical results were in error to a similar extent and did not trigger a delta alert.

Platelets usually are counted by impedance analysis or by optical procedures (refractive and laser light-scattering properties). The CELL-DYN CD4000 system (Abbott Laboratories) simultaneously provides both and offers a further optional approach using immunoplatelet analysis (2, 3). In the latter method, a fluorescein isothiocyanate-labeled monoclonal antibody (CD61; Clone RUU-PL 7F12, IgG1 subclass) binds the glycoprotein (Gp)Illa part of the GpIb/IIa receptor subunit (3). This glycoprotein is expressed by all human platelets, both resting and activated, but not by erythrocytes or leukocytes. The immunochemical platelet count is largely unaffected by sample interferences and allows determination of platelet counts to $<1 \times 10^9/L$ (4). The analytical performance is virtually identical to that reported for alternative flow cytometric immunomethods (5).

We recently encountered four patients with cryoglobulinemia in whom erroneously high platelet counts were obtained by both optical and impedance platelet counts. Patient 1 had lymphoplasmacytoid lymphoma. Because the CD4000 analyzer flagged the presence of variant lymphocytes, a peripheral blood smear was examined. This confirmed the presence of lymphoma cells and also revealed thrombocytopenia, whereas the automated impedance and optical analyses suggested platelet counts of $\sim 355 \times 10^9/L$ (Table I). The result from a chamber (hemacytometry) count ($38 \times 10^9/L$) also indicated thrombocytopenia, and a CD4000 CD61 immunoplatelet count ($42 \times 10^9/L$) further substantiated the morphologic conclusions. Therefore, both the impedance and optical platelet counts were overestimated and, because the errors were of similar numeric magnitude, no CD4000 delta alert was given. Consequently, this patient’s thrombocytopenia would have been missed had the manual slide review not been performed for other reasons. We processed these same samples with other hematology analyzers (Sysmex SE8000, Sysmex K4500, Sysmex XE2100, and Bayer Technicon H*3) and found platelet count overestimates similar to that of the CD4000.

Patient 2 (Table I) had Waldenström macroglobulinemia, but at the time of CBC analysis he was not thrombocytopenic. Both the CD4000 (impedance and optical) and Technicon H*3 (optical) instruments significantly overestimated the platelet count. Two other patients seen during the past 18 months demonstrated similar patterns of interference. Both had cryoglobulinemia, one associated with centroblastic lymphoma (patient 3) and the other with Waldenström macroglobulinemia (patient 4; see Table I). These patients also had several episodes of thrombocytopenia during their hospital stays for cytoreductive chemotherapy.

Because of these observations, we wished to develop a review procedure for recognizing such situations in the absence of a significant difference between CD4000 impedance and optical platelet counts. Frequency distributions of platelet size may show increased steepness or asymmetry in the lower ends of platelet size histograms.