Successful Diagnosis of Fetal Gender Using Conventional PCR Analysis of Maternal Serum

HIROSHI HONDA, NORIO MIHARU, YOKO OHASHI, and KOSO OHAMA

Background: Fetal DNA has been found in maternal plasma and serum. Diagnosis of fetal gender using maternal plasma and serum has been attempted in an effort to develop a new noninvasive method of prenatal diagnosis.

Methods: Peripheral blood samples were obtained from 61 pregnant women at 10–17 weeks of gestation before amniocentesis. DNA was extracted from 800 μL of each plasma or serum sample. To detect the Y-chromosome-specific sequences DYS14 and DYZ3 in the maternal plasma and serum, 40 cycles of PCR were carried out for each DNA extract. The PCR products were analyzed by 2.5% agarose gel electrophoresis and ethidium bromide staining, and the results were compared with the results of the cytogenetic analyses of amniocentesis.

Results: Cytogenetic analysis of amniocentesis revealed that 31 pregnant women had a male fetus and the remaining 30 pregnant women had a female fetus. Both DYS14 and DYZ3 were detected in 27 of the 31 plasma samples obtained from pregnant women carrying a male fetus and in all of 31 serum samples obtained from the same women. Neither DYS14 nor DYZ3 was detected in either the plasma or serum samples obtained from any of the 30 pregnant women carrying a female fetus.

Conclusion: PCR analysis of maternal serum can be used to diagnose fetal gender.

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The use of fetal cells, such as nucleated red blood cells (NRBCs), has been attempted as a means of noninvasive prenatal diagnosis. To date, this noninvasive diagnostic method has not been made practicable, although intensive studies have been performed. Apparently the very small amount of fetal cells in maternal blood and difficulties in isolating the fetal cells from maternal blood are limitations that have not been overcome. In fetal gender diagnosis using enriched fetal NRBCs, the sensitivity of detecting a male-bearing pregnancy has been reported to be 55–86% (1–3). Thus, the gender of a fetus cannot be diagnosed exactly with this method.

Lo et al. (4) showed the presence of fetal DNA in maternal plasma and serum. These researchers also showed that fetal DNA is present in the total DNA in maternal plasma and serum at concentrations much higher than the number of NRBCs found in the maternal blood cells in whole blood (5). Lo et al. (5) documented that during early pregnancy, fetal DNA concentrations were, on average, 3.4% of the total DNA in the maternal plasma and 0.13% in serum.

We diagnosed fetal gender using conventional PCR on DNA solutions extracted from the maternal plasma and serum. This method for diagnosing fetal gender can be used as a pre-test to determine whether invasive prenatal diagnoses, such as amniocentesis and chorionic villi sampling, should be performed on a fetus having a risk of X-linked recessive inheritance. Our results also indicated that we can accurately detect fetal DNA; thus, fetal DNA in maternal plasma and serum may be considered a new material for noninvasive prenatal diagnosis.

Materials and Methods

Sample Preparation
Peripheral blood samples were obtained from 61 pregnant women undergoing amniocentesis at Hiroshima University Hospital during the period from June 1998 to March 1999. All of the pregnant women participating in this study were selected at random and gave informed written consent before blood sampling. All of the pregnant women underwent blood sampling before amniocentesis. At the time of blood collection, the gestational ages ranged from 10 weeks + 5 days to 17 weeks + 3 days (mean, 13 weeks + 2 days; Fig. 1).

Peripheral blood samples obtained from five healthy men and five nonpregnant women were used to determine the PCR sensitivity and as negative controls, respectively. In each case, 4 mL of peripheral blood was collected into an EDTA-containing Vacutainer Tube for
plasma separation, and 6 mL of peripheral blood was collected into a Vacutainer Tube containing no anticoagulant for serum separation. The blood samples were centrifuged at 3000g, and the plasma and serum were carefully removed from their respective tubes and transferred into plain polypropylene tubes. The plasma and serum samples were again centrifuged at 3000g, and these recentrifuged plasma and serum samples were transferred into fresh polypropylene tubes. The samples were stored at −20 °C until further processing.

**DNA extraction from plasma and serum samples**

DNA was extracted from the plasma and serum samples by the QIAamp DNA Blood Mini method (Qiagen) according to the "blood and body fluid protocol" with minor modifications. We extracted the DNA from 800 µL of plasma and serum in each case. The volume of the extracted DNA solution was usually 200 µL, and the extracted DNA solution was then concentrated to a volume of 20 µL by ethanol precipitation. Each DNA solution extracted from the healthy men was serially diluted from 1:10 to 1:10,000.

**PCR**

The Y-chromosome-specific sequences DYS14 and DYZ3 were amplified for the detection of fetal DNA in maternal plasma and serum. We used the primer sets Y1-7/Y1-8 (6) and Y1-1/Y2-2 (7) to amplify DYS14 and DYZ3, respectively. For DYS14, PCR amplification was performed in a total volume of 25 µL containing extracted DNA, 200 µM dNTPs, 20 pmol of each primer (Y1-7/Y1-8), 1× Taq polymerase buffer (containing 1.5 mM MgCl₂), and 0.75 U of Taq polymerase; the PCR product was 172 bp. The thermal cycling for DYS14 was as follows: denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 57 °C for 1 min, and 72 °C for 2 min, with final incubation at 72 °C for 7 min. For DYZ3, the thermal cycling began with denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 40 s, 55 °C for 30 s, and 72 °C for 1 min, with final incubation at 72 °C for 7 min. The PCR amplification products were separated by 2.5% agarose gel electrophoresis and visualized by exposure to ultraviolet light after ethidium bromide staining.

The samples were coded, and the PCR analysis was always performed before cytogenetic analysis of cultured amniocytes. The PCR results were compared with the fetal gender revealed by the cytogenetic analysis. The individuals performing or interpreting the cytogenetic analysis did not know the PCR results. Each DNA solution extracted from the plasma and serum of pregnant women, healthy men, and nonpregnant women was tested three times in different PCR sessions. Any samples that tested positive at least twice among the three amplifications were considered positive. If the result for a DNA extract from either the plasma or serum of the pregnant women, the nonpregnant women, or the healthy men was negative, the DNA extracts from the plasma and its paired serum sample or the serum and its paired plasma sample were further amplified for an autosomal locus, glyceraldehyde-3-phosphate (GAPDH), to assess the presence of DNA.

**Anticontamination measures**

Great care was taken to prevent PCR contamination (8). Aerosol-resistant pipette tips were used for all liquids. Separate areas were used for the extraction of DNA, the preparation of amplification reactions, the carrying out of amplification reactions, and the detection of the PCR products, and all manipulations except the detection were carried out in a laminar flow hood.

**Results**

In the dilution series prepared with DNA extracted from the plasma and serum of all five healthy men, the detection limits were the 1:1000 dilution for DYS14 and the 1:100 dilution for DYZ3 (Fig. 2). Neither DYS14 nor DYZ3 was detected in the DNA extracted from any of the five nonpregnant women.

Cytogenetic analysis of cultured amniocytes revealed that 31 of the pregnant women were carrying a male fetus and the remaining 30 pregnant women were carrying a female fetus (Table 1). Both DYS14 and DYZ3 were detected in all three PCR analyses in the plasma samples from 27 of the 31 male-bearing pregnant women. Of the four remaining male-bearing pregnant women, the DYS14 sequence but not the DYZ3 sequence was detected one time in one woman, and neither the DYS14 nor the DYZ3 sequence was detected in the plasma of the remaining three women. In the serum samples from the 31 male-
bearing pregnant women, both the DYS14 and DYZ3 sequences were detected in all three PCR analyses in all of the subjects. Thus, of the 31 women identified as carrying a male fetus by cytogenetic analysis, our plasma-based method indicated that 27 were carrying a male fetus, whereas our serum-based method indicated that all 31 were carrying a male fetus (Fig. 3 and Table 2). The four male-bearing pregnant women for whom the plasma samples had been negative had had their blood collected early in the gestational period (11–13 weeks of gestation). Neither the DYS14 nor the DYZ3 sequence was detected in either the plasma or serum of any of the 30 female-bearing pregnant women. We were thus able to determine by fetal gender diagnosis using maternal plasma or serum that all 30 of these pregnant women were carrying a female fetus (Fig. 3 and Table 2).

The GAPDH sequence was detected in the undiluted DNA solutions from the plasma and serum of the 4 male-bearing pregnant women for whom the gender of the male fetus could not be determined from maternal plasma, the 30 female-bearing pregnant women, and the 5 nonpregnant women (Table 3). In the 1:10 dilutions of DNA from plasma, the GAPDH sequence was detected in 28 of the female-bearing pregnant women and the 1 male-bearing pregnant woman in whom the DYS14 sequence was detected in only one PCR analysis and the DYZ3 sequence was not detected; however, in the 1:10 dilutions of DNA from serum, the GAPDH sequence was detected in all 30 of the female-bearing pregnant women and all 4 of the male-bearing pregnant women for whom the gender of the male fetus could not be determined from maternal plasma. The GAPDH sequence was also detected in the 1:10 dilutions of DNA from both the plasma and serum of the five nonpregnant women. However, GAPDH was not detected in the plasma samples of three of the four male-bearing pregnant women for whom the gender of the male fetus could not be determined from maternal plasma, and in two female-bearing pregnant women (Table 3).

### Table 1. Cyogenetic analysis of cultured amniocytes.

<table>
<thead>
<tr>
<th>Karyotype</th>
<th>n</th>
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<tr>
<td>46,XY</td>
<td>31</td>
</tr>
<tr>
<td>46,XX</td>
<td>28</td>
</tr>
<tr>
<td>mos 46,XX/47,XX+21</td>
<td>1</td>
</tr>
<tr>
<td>45,XX, +der(13,14)(q10;q10)</td>
<td>1</td>
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**Discussion**

We performed fetal gender diagnosis using a Y-chromosome-specific sequence in maternal whole blood or in enriched fetal NRBCs. The sensitivity of the nested PCR for detecting male-bearing pregnancies from maternal whole blood at 10–18 weeks of gestation was 83–94% (9–11), whereas the sensitivity of fluorescence in situ hybridization or PCR using enriched fetal NRBCs ranged...
from 55% to 86% (1–3). However, these methods are time-consuming and labor-intensive, and their sensitivities are quite low for a clinical application. On the other hand, when we used maternal serum, our method had the highest sensitivity (100%) reported to date, and with maternal plasma, our method had a sensitivity (87%) comparable to the previously mentioned methods. Our method is based on the presence of fetal DNA in maternal plasma and serum, a finding that was reported for the first time by Lo et al. (4). The high sensitivity for detecting fetal DNA with conventional PCR is attributable to a high concentration of fetal DNA in maternal plasma and serum. The detection of both DYS14 and DYZ3 sequences in the DNA extracted from the serum of male-bearing pregnant women indicates that the fractional concentration of fetal DNA in maternal serum is >0.1%, based on the assumption that the total DNA present in male serum is equal to that in maternal serum and that there is no difference in the number of repetitive sequences of DYZ3 among individuals. This concentration is at least 385-fold higher than the concentration reported in the cellular fraction (2.6 × 10^-6%) by Hamada et al. (12). In fact, Lo et al. (5), using real-time quantitative PCR (TaqMan PCR), showed that the fractional concentration of fetal DNA in maternal serum in early pregnancy (11–17 weeks of gestation) is 0.014–0.54% (mean, 0.13%). This concentration is similar to that in enriched fetal NRBCs (0.001–5%) in all cellular fractions after fluorescence-activated cell sorting (13).

The accuracy of our method for diagnosing fetal gender was significantly higher when we used maternal serum rather than maternal plasma (P < 0.0387, χ² analysis). Because there theoretically is no difference between the concentration of fetal DNA in maternal plasma and serum, the difference in the sensitivity for detecting fetal DNA between plasma and serum might reflect (a) a DNA extraction efficiency that is higher for serum than for plasma, or (b) a fetal DNA concentration in maternal plasma at early gestational ages that is below the detection limits for the DYS14 and DYZ3 sequences.

Regarding the efficiency of DNA extraction, to estimate extraction efficiency, we analyzed the concentration of GAPDH, a housekeeping gene. In three of four male-bearing pregnant women for whom the plasma results were incorrect, GAPDH was not detected in the 1:10 dilutions of DNA extracted from their plasma samples but was detected in the 1:10 dilution of DNA extracted from their serum samples. This result indicates a lower efficiency of DNA extraction from plasma than from serum. In the present study, this lower extraction efficiency might be attributable to cryoprecipitates in the plasma. The manufacturer’s manual for the QIAamp DNA Blood Mini method indicates that cryoprecipitates may interfere with DNA extraction (14).

Regarding the second possibility, i.e., that low concentrations of fetal DNA in maternal plasma may lead to incorrect diagnosis of fetal gender, the maternal plasma samples in our study for which the gender of the male fetus was incorrectly diagnosed were collected at an early gestational age (11–13 weeks). Because the concentration of fetal DNA in maternal plasma or serum increases with gestational age (5), incorrect diagnosis of fetal gender based on maternal plasma might be attributable to the lower concentration of fetal DNA at early gestational ages compared with late gestational ages. However, fetal gen-

**Fig. 3.** Detection of DYS14 (A) and DYZ3 (B) sequences in maternal plasma and serum by PCR.

Lanes 1 and 12, 100-bp DNA size ladder; lanes 2, 4, and 6, DNA solutions from the plasma of male-bearing pregnant women amplified for the DYS14 sequence; lanes 3, 5, and 7, DNA solutions from the serum of male-bearing pregnant women amplified for the DYS14 sequence; lanes 8 and 9, DNA solution from the plasma and serum, respectively, of female-bearing pregnant women amplified for the DYS14 sequence; lanes 13, 15, and 17, DNA solutions from the plasma of male-bearing pregnant women amplified for the DYZ3 sequence; lanes 14, 16, and 18, DNA solutions from the serum of male-bearing pregnant women amplified for the DYZ3 sequence; lanes 19 and 20, DNA solution from the plasma and serum, respectively, of female-bearing pregnant women amplified for the DYZ3 sequence; lanes 10 and 21, distilled water without DNA that underwent PCR for DYS14 and DYZ3, respectively; lanes 22, and 11, diluted DNA solution extracted from male whole blood amplified for the DYS14 and DYZ3 sequences, respectively.
sensitivity for detecting viral or fetal DNA suggests that the extraction efficiencies for plasma and serum might differ according to the DNA extraction method used. According to Dixon et al. (19), the QIAamp DNA Blood reagent set is one of the best methods for extracting DNA from plasma or serum. Because this DNA extraction method is more efficient for serum than for plasma, we believe that maternal serum is more suitable than plasma for fetal gender diagnosis.

Among Y-chromosome-specific sequences, including DYZ1, sex-determining region Y (SRY), zinc finger protein, Y-encoded (ZFY), and the amelogenin-like gene on Y chromosome (AMELY), we chose the DYS14 and DYZ3 sequences for diagnosis of fetal gender because DYS14 has been used frequently in the past, and DYZ3, which has tandem repeat units, was suspected to be highly sensitive in the detection of male fetal DNA. The detection limit for the DYS14 sequence was lower than that for DYZ3 in our PCR conditions; therefore, the sensitivity of DYS14 for the detection of male fetal DNA is potentially higher than that of DYZ3. However, in the present study, the sensitivity of the DYS14 sequence for the detection of fetal male DNA was the same as that of the DYZ3 sequence in both maternal plasma and serum at 10–17 weeks of gestation. These results depend on the concentration of male fetal DNA being higher than the detection limit of DYZ3 in all of the cases. Because the concentration of male fetal DNA usually increases with gestational age (5), it is likely that the sensitivity of the DYS14 sequence for detecting fetal male DNA may be higher than that of the DYZ3 sequence at an early gestational age.

Because we successfully used maternal serum to diagnose fetal gender, our diagnostic method may be useful in place of invasive prenatal diagnostic methods such as amniocentesis and chorionic villi sampling in cases of X-linked recessive inheritance. If a fetus has a risk of X-linked recessive inheritance and is found to be female by our maternal serum-based method, use of an invasive

| Table 2. Comparison of the results of PCR and cytogenetic analysis. |
|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
|                      | DYS14 (+)             | DYS14 (-)             | DYZ3 (+)              | DYZ3 (-)              |
| Fetal gender *       | Plasma (n = 31)       | Serum (n = 30)        | Plasma (n = 27)       | Serum (n = 30)        |
| Male                  | 27                    | 31                    | 4                     | 0                     |
| Female               | 0                     | 0                     | 30                    | 30                    |

* Determined by cytogenetic analysis.

| Table 3. Results of the amplification of a series of diluted DNA solutions for GAPDH sequence. |
|----------------------------------------|-----------------|-----------------|-----------------|
| Dilution series                        | Plasma (n = 4)  | Serum (n = 30)  | Plasma (n = 5)  | Serum (n = 5)  |
| Undiluted DNA solution                 | 4               | 30              | 5               | 5               |
| DNA solution diluted 1:10              | 1              | 28              | 5               | 5               |

* Male-bearing pregnant women for whom the gender of the male fetus could not be determined by fetal gender diagnosis using maternal plasma.

* Male-bearing pregnant women in whom the DYS14 sequence was detected once and the DYZ3 sequence was not detected at all.
prenatal diagnostic method may not be necessary. Our success in diagnosing fetal gender shows that we can easily detect fetal DNA extracted from plasma or serum. Therefore, we can also use fetal DNA obtained from maternal serum in the prenatal diagnosis of other paternally inherited autosomal-dominant diseases, of diseases caused by germline mutations, and of fetal RhD status (20). With quantitative analysis of fetal DNA, we may also assess the prognosis for pregnancies in which abnormal conditions such as preeclampsia and threatened premature delivery are present, as well as the presence of fetal chromosomal abnormalities (21–23). In the near future, maternal plasma and serum may play an important and powerful role in noninvasive prenatal diagnosis.

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References


